Author:
Tasman, Kiah

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The effect of neonicotinoid pesticides on the circadian clock and sleep of fruit flies and bumblebees

The effect of neonicotinoid pesticides on the circadian clock and sleep of fruit flies and bumblebees

Kiah Tasman

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Abstract

Neonicotinoid insecticides are agonists of the nicotinic acetylcholine receptors that mediate excitatory, fast synaptic transmission throughout the insect nervous system. Due to their environmental prevalence and the involvement of the nicotinic acetyl choline receptors in many behaviours, neonicotinoids are a major factor in insect declines.

Many sub-lethal effects have previously been identified for neonicotinoids in beneficial insects. Here, the effects of four neonicotinoids, imidacloprid, clothianidin, thiamethoxam and thiacloprid, on the insect clock and sleep are characterised for the first time. Drosophila was used as a model, allowing rapid assessment of behavioural effects and investigation into the mechanism of action on the clock. Imidacloprid, clothianidin and thiamethoxam all disrupted locomotion and circadian rhythmicity and fragmented and reduced sleep in Drosophila. Thiacloprid only affected sleep.

Nicotinic acetylcholine receptor subunits Dα1, Dα3 and Dβ2 were then knocked down in the clock neurons. These knockdowns showed the same disruptions to circadian rhythmicity and sleep as seen in neonicotinoid exposed flies, suggesting these subunits are involved in sleep and circadian behaviour. Exposure of these flies to imidacloprid or clothianidin had no further effect on rhythmicity, suggesting that neonicotinoids act upon the clock neurons to disrupt rhythmicity and that Dα1, Dα3 and Dβ2 mediate this effect. Exposure to imidacloprid or clothianidin or knockdown of Dα1, Dα3 and Dβ2 prevented the circadian remodelling of the dorsal terminals of the s-LNv clock neurons, further suggesting that neonicotinoids may act directly upon the clock neurons.

Behavioural assays were repeated in the buff tailed bumblebee *Bombus terrestris*. Foragers showed a reduction in locomotor and foraging rhythmicity. They also showed greatly reduced foraging activity and an increase in daytime sleep. Reduced foraging activity and rhythmicity is likely to reduce the capacity of colonies to grow and reproduce. This could be a contributary factor in insect declines in the wild.

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Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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List of Abbreviations

ACh- Acetylcholine

ANOVA - Analysis of Variance

BEETag - Behavioural ecology tag

CLK - Clock

CNS - Central Nervous System

CRY - Cryptochrome

CYC - Cycle

DD - Constant Darkness

DAM - *Drosophila* activity monitor

DART - *Drosophila* Arousal Tracker

DN - dorsal neurons

DDT - Dichlorodiphenyltrichloroethane

GFP - Green fluorescent protein

HB-eyelet - Hofbauer-Buchner eyelet

LAM - Locomotor Activity Monitor

LD - 12:12 light-dark cycle

l-LNv - Large ventral lateral neuron

s-LNv - Small ventral lateral neuron

LNd - dorsal lateral neuron

nAChR - nicotinic acetylcholine receptor

PDF - Pigment dispersing factor

PER - Period

PFA - paraformaldehyde

PI - *pars intercerebralis*

PL - *pars lateralis*

REM - Rapid Eye Movement

RFID - Radio Frequency Identification

RNAi - RNA interference

RS - Rhythmicity statistic

SCAMP- Sleep and Circadian Analysis MATLAB Program

SEM - Standard error of the mean

TIM - Timeless

UAS - Upstream-activating sequence

UV - Ultraviolet

ZT - Zeitgeber time

GENE AND PROTEIN NOMENCLATURE

Following convention gene symbols are lowercase italicised and protein symbols are uppercase regular type, (e.g. per/PER).

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Chapter 1 Introduction

"If all mankind were to disappear, the world would regenerate back to the rich state of equilibrium that existed ten thousand years ago. If insects were to vanish, the environment would collapse into chaos"

E. O. Wilson[1]

We are currently living through the sixth mass extinction event, the 'anthropocene extinction'[2]. Human activity and the associated changes in habitat availability and quality are causing mass reductions in biodiversity and abundance. Over the past fifty years, insects (the most abundant and varied class of animal) have experienced significant reductions, documented by multiple longitudinal studies. In Germany, there was a 76% decrease in flying insect biomass between 1989 and 2016[3]. A study in Puerto Rico found that between 1976 and 2012, 96% of ground insects had disappeared, causing reductions in other groups such as birds and frogs[4]. Globally, it is estimated that 40% of insect species are at risk of extinction, with total insect biomass decreasing by 2.5% every year[5]. As seen in Puerto Rica, insects are a vital food source, endangering all species who appear higher than them on the food chain. They also provide many ecosystem services such as pest control, decomposition and pollination[6]. Insect pollination is required by 80% of wild plants, and 75% of crops and is worth over €153 billion to agricultural markets worldwide[7-9]. Most pollination is carried out by bees, which are one of the insect groups most heavily affected by population losses[5]. Many factors are contributing to these losses, including climate change, habitat loss and invasive species, but one of the major causes is the intensive use of insecticides in agriculture[5]. Aimed at killing pest species, insecticides are non-specific, affecting beneficial insects in the same way as pests. Globally, the most commonly used insecticides are the neonicotinoids, which have a proven range of lethal and sub-lethal effects on beneficial insects such as bees, and are a major factor in their decline[10].

In this thesis, I have characterised the effects of neonicotinoid pesticides on a previously unexplored area: circadian rhythms and sleep. Circadian rhythmicity is vital to the function of organisms and influences a diverse range of behaviours in insects, such as foraging, pollination, communication, mating, egg laying, and sleep, which in turn is necessary for memory consolidation[11-16]. The disruptions to the clock and sleep identified in this thesis add to the heavy weight of existing evidence of the harm of neonicotinoid pesticides to beneficial insects.

1.1 Neonicotinoid insecticides

Neonicotinoid insecticides are chemically similar to nicotine, which has been used as a pesticide in the form of tobacco plantations since at least 1690[17]. The first commercial neonicotinoid, imidacloprid, was patented by Bayer in 1985 and brought to market in 1991[17,18]. Since then their popularity has grown rapidly, overtaking previously wide-spread pesticides such as organophosphates. Neonicotinoids gained popularity due to their high potency, their long-lasting effects and their systemic nature[19]. This provides protection to all parts of the plant, making neonicotinoids effective against boring insects such as the emerald ash borer (*Agrilus planipennis*), root feeding insects such as the western corn rootworm (*Diabrotica virgifera virgifera*) and sap sucking insects such as aphids [20-22]. They are currently the most commonly used insecticides in the world, making up 24% of the total agrochemical market and 80% of the seed treatment market[23]. Since the launch of imidacloprid, many other popular neonicotinoids have come onto the market, including clothianidin, thiamethoxam and thiacloprid.

Neonicotinoids function as insecticides *via* their agonistic action at nicotinic acetylcholine receptors (nAChRs)[24]. These receptors are found throughout the insect central nervous system, and their natural agonist, acetylcholine (ACh) is the main excitatory neurotransmitter in the insect brain[25]. When neonicotinoids bind to nAChRs, they can cause initial depolarisation of the cell, activating voltage-sensitive Na^+ channels which mediate action potentials. Sustained activation of the nAChRs by the agonist can result in voltage-dependant inactivation of Na+ channels and desensitisation of the nAChR, resulting in a depolarising block and causing functional inactivation of the cell[24].

1.1.1 Routes for Exposure

Neonicotinoids are used as a traditional spray insecticide but also make up the majority of the seed treatment market[23]. Due to their high solubility and their systemic nature, neonicotinoids are absorbed by treated plants and appear in every tissue[26]. Whilst this increases the insecticides' efficacy against sap sucking pests[27], it also means that the pollen and nectar of the plant contains significant concentrations of neonicotinoid. Analysis of crops has shown that nectar and pollen

contain 1-51 µg/L for seed treated crops and 61-127 µg/L for sprayed crops[19]. These concentrations are sufficient to cause sub-lethal effects in non-target species; a dose of 1 µg/L of imidacloprid can reduce brood production by a third in *Bombus terrestris (B. terrestris)*[28]. Insects can be exposed to higher doses shortly after spraying or when treated seeds are being sown, due to high concentration neonicotinoid dust and particulates being blown into the air[29]. In fact, the majority of the neonicotinoid does not end up in the target plant. Up to 95% ends up in the surrounding environment, being either blown away or washed into soil and water sources[26,30], often ending up in the pollen and nectar of wild, untreated plants[31] (Fig. 1.1). Although broken down fairly quickly by ultraviolet (UV) exposure, in the soil neonicotinoids can have a half-life of over 3 years and many of their metabolites are also toxic[26], leading to ongoing exposure long after their initial use. One study carried out in the UK found that neonicotinoid concentrations in the soil of farmland annually planted with seed treated wheat increased by approximately 10 µg/L (or 10 parts per billion (ppb)) every year[19].

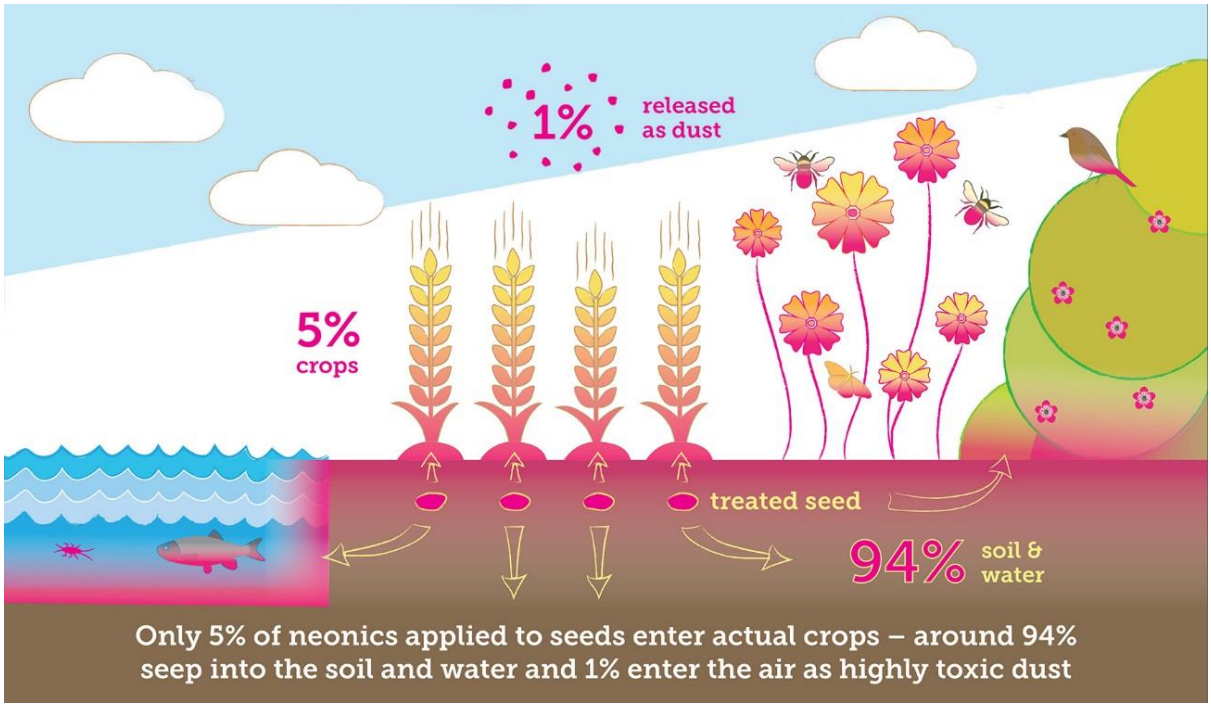

Figure 1.1- The occurrence of neonicotinoids in the environment

An illustration of the proportion of neonicotinoid pesticides that end up in the treated crop, compared to the proportion that ends up in the environment.

Figure from the Soil Association[32].

1.1.2 Effects on non-target species

Due to the importance of nAChRs in the insect nervous system and the high efficacy of neonicotinoids, as potent neurotoxins they have a wide range of harmful effects on non-target insects. The acute lethal dose of neonicotinoids can be remarkably low, *e.g.* 0.018 µg per bee for imidacloprid[33] and is half that of pest species such as aphids[34,35]. Pollinators can experience lethal doses of neonicotinoids in the field, particularly during spraying or sowing of treated crops. However, even at the sub-lethal doses commonly found in nectar, insects can experience a variety of behavioural disruptions.

Studies looking at the effects of field-realistic doses of neonicotinoids in honeybees and bumblebees have found that they can disrupt behaviours including learning and memory[36,37], locomotion[36], foraging success[38], foraging motivation[39] and grooming behaviour[40]. These behavioural effects at the individual level appear to culminate in reduced reproductive success at the colony level. Neonicotinoid exposure has been shown to lead to slower colony growth in bumblebees, resulting in an 85% reduction in queen production[41]. Neonicotinoids also appear to directly affect the fertility of reproductive individuals, and are capable of causing a 39% reduction in living sperm in male honeybees[42] and delay egg laying in bumblebee queens[43]. Neonicotinoids can also affect brain development and neuron health. In honeybees, neonicotinoid exposure has been shown to increase apoptosis[44] and mitochondrial dysfunction[44] of neurons in the brain. In stingless bees, larval neonicotinoid exposure can reduce mushroom body mass by up to 36% in the resulting adult bees[45].

It has previously been suggested that these effects are unlikely to occur in the field because insects have a choice of food and will avoid plants treated with neonicotinoids. However, work using *B. terrestris* has since shown that neonicotinoids are actually attractive to insects[46]. Additionally, although most studies and this thesis focus on the effects of exposure to individual neonicotinoids, it is worth bearing in mind that in the field, insects often experience combined exposure to multiple pesticides[47], alongside environmental stressors such as pests and disease, so harmful effects observed in controlled, single exposure studies may reflect more severe effects in the field[40,48-50].

The detrimental effects on insects can have knock on consequences for reliant species. In the UK and the Netherlands, the insect decline has occurred alongside a parallel reduction in insect-pollinated plants[51]. Further, as seen in Puerto Rico, the global decline in insect populations is depriving many species of a valuable food source, resulting in further declines[4]. Neonicotinoids can also accumulate in larger animals feeding on contaminated insects and seeds, such as birds[52]. This exposure can lead to reduced food consumption, body mass, and delayed migration in song birds, which can in turn lead to reduced survival and reproductive success[53-55]. Thus, neonicotinoid exposure is likely to be a

contributing factor to declines not only in insect populations, but also animals further up the food chain such as birds, providing a parallel with the 'silent spring's which characterised the years of the pesticide Dichlorodiphenyltrichloroethane (DDT)'s popularity[56].

1.1.3 European Union ban

In 2012 the EU approved five neonicotinoid pesticides for use: imidacloprid, clothianidin, thiamethoxam, thiacloprid and acetamiprid[57]. Due to the mounting evidence of the potential harm these insecticides posed to beneficial insects, in 2013 the EU placed a moratorium on the use of imidacloprid, clothianidin and thiamethoxam, severely restricting their use in the field[58]. In 2018 it was voted that this ban should become permanent and should be extended to cover all field crops. Acetamiprid and thiacloprid are still approved for use, although thiacloprid is currently categorised as 'candidate for substitution', meaning that the EU mandates national authorities to attempt to find valid alternatives. The status of thiacloprid as an approved substance in currently under review and will be voted on by EU member states at the end of 2019[59]. The approval for acetamiprid has already been renewed until 2033 due to 'low risk to bees'[60].

Despite the moratorium and resultant ban, there are still routes for exposure for beneficial insects in the EU. Neonicotinoids can still be used in greenhouses and gardens and persist in the environment. Analysis of garden plants being sold as 'bee friendly' in the UK in 2017 found that 70% of them had been treated with neonicotinoids[61]. Another study compared the exposure of bumblebees to neonicotinoids the year before and the year after the moratorium came into action in the UK in 2014. They found that there was no significant reduction in the residue concentrations of banned neonicotinoids in nectar collected from bumblebee colonies for either rural or peri-urban environments but that there was a significant increase in thiacloprid concentrations after the moratorium[62]. Other research in the UK found that over 20% of honey samples collected a year after the moratorium contained banned neonicotinoids[63]. The highest concentrations of neonicotinoids were found in honey produced near oil seed rape plantations and during the flowering season, suggesting that despite not having been treated, these crops contained neonicotinoids due to ongoing environmental contamination.

The continued neonicotinoid ban is popular with the public, and nearly five million people signed a petition for the EU to extend the ban in 2018. However, it is possible that post-Brexit, policy on neonicotinoid use in the UK will change as we will no longer be bound by the ban. The UK government have already granted multiple exceptions to the ban, allowing use of neonicotinoids on oil seed rape in certain areas of the UK[64]. Analysis of the manner by which current EU pesticide legislation will be brought into UK law shows a significant erosion of environmental protections[65].

Currently, independent scientific advice must be sought on pesticide approval; however, post - Brexit, ministers may choose whether or not to consider scientific evidence at their own discretion. The proposed legislation also provides the UK heads of state full power to 'amend, revoke, make regulations and issue guidance on implementation' for policy on pesticide use. There is precedent for revocation of pesticide bans; in 2014, the USA banned the use of neonicotinoids in wildlife refuges[66]. However, in 2018 under the Trump administration, this ban was overturned[67].

1.1.4 Current and future pesticides utilising the same target site

Despite the controversy surrounding the use of neonicotinoids, they remain the most commonly used insecticides across the globe and the nAChR as a target site is proving fertile ground for novel pesticides. Many analogous pesticides are currently being synthesised and tested that utilise the same mechanism of action[68]. One, spinosad, is authorised for use in the EU and has been approved for use on organic crops[69], despite evidence that the toxicity of spinosad to honeybees may be similar to that of imidacloprid[70]. Therefore, ongoing research into the full range of effects that insecticides targeting the nAChR can have is warranted.

1.2 The nicotinic acetylcholine receptor in insects

1.2.1 Structure and conservation of the nicotinic acetylcholine receptor

The insect nAChR is a pentameric ligand gated ion channel, composed of five nAChR subunits, forming a complex with a pore in the centre[71]. When open, this channel allows the movement of Na^+, K^+ and often Ca^{2+} across the membrane, allowing rapid depolarisation in membrane potential at normal resting membrane potentials[72]. The nAChR subunits are divided into α and β types, and assemble to produce either α subunit homomeric nAChRs, α subunit heteromeric nAChRs or α and β subunit heteromeric nAChRs[71]. An nAChR subunit consists of 4 transmembrane domains and an N-terminal extracellular domain with a characteristic Cys-loop that aids in receptor assembly and ion channel gating[73] (Fig. 1.2). The binding site of the nAChR is formed by 6 regions at the juncture between two subunits; the A-C loops of an α subunit and the D-F loops of either an α or a β subunit[73]. The presence of 2 adjacent cysteine loops in the C loop is important for ACh binding and is what distinguishes an α type subunit.

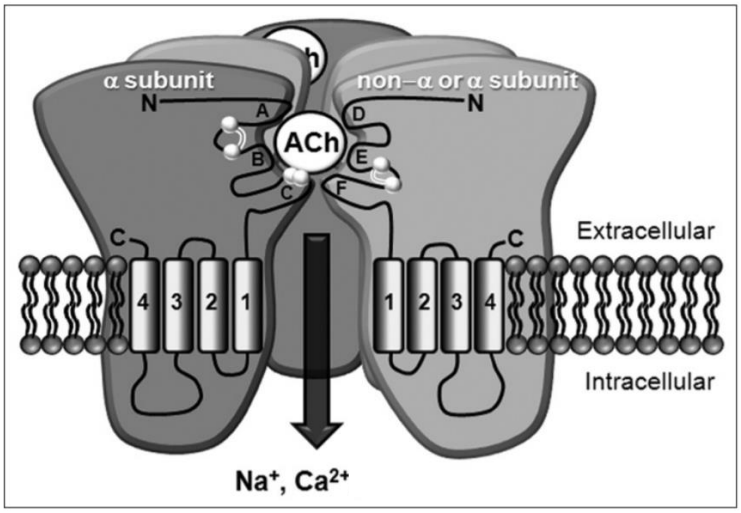

Figure 1.2 The insect nicotinic acetylcholine receptor

An illustration of the insect nAChR, which can take the form of either a homomeric receptor formed of five α subunits or a heteromeric receptor consisting of a mix of both α and β subunits. Within the subunits can be seen the 4 transmembrane domains and the 6 regions (loops A-F) which form the binding site. Figure from Jones & Sattelle[73].

The subunit composition of the nAChR dictates its functional and pharmacological properties, producing a diverse number of nAChR subtypes. *Drosophila melanogaster* was the first insect in which all of the nAChR subunits were described[72]. There are ten subunits in *Drosophila*, Dα1-7 and Dβ1-3[71]. Following nAChR subunit discoveries in other species have been grouped with regards to their homology to the *Drosophila* subunits (Fig. 1.3). It appears that the nAChR subunits of most insects, including honeybees can be placed into seven highly homologous groups, each with over 60% amino acid identity[72]. Each insect also appears to have a repertoire of distinct nAChR subunits, such as the Dβ3 subunit in *Drosophila*.

Current knowledge of the subunit composition and functional role of different nAChR subtypes is limited. The creation of functional heterologous insect nAChRs has proven difficult, leaving us reliant on co-localisation of expression patterns, immunoprecipitation experiments and the creation of chimeric receptors using both invertebrate and vertebrate subunits[72]. Based on data from these assays, one group put forward three likely nAChR compositions; one involving at least Dβ1 and Dβ2 and an α subunit, one containing at least Dα1, Dα2 and Dβ2 and one with at least Dβ1 and Dα3[74]. Whilst we know that only some nAChR subtypes are susceptible to neonicotinoids[75], the composition of these subtypes has not been shown. However, exploration of neonicotinoid resistance using

Drosophila as a model has identified some nAChR subunits which appear to be involved in susceptibility.

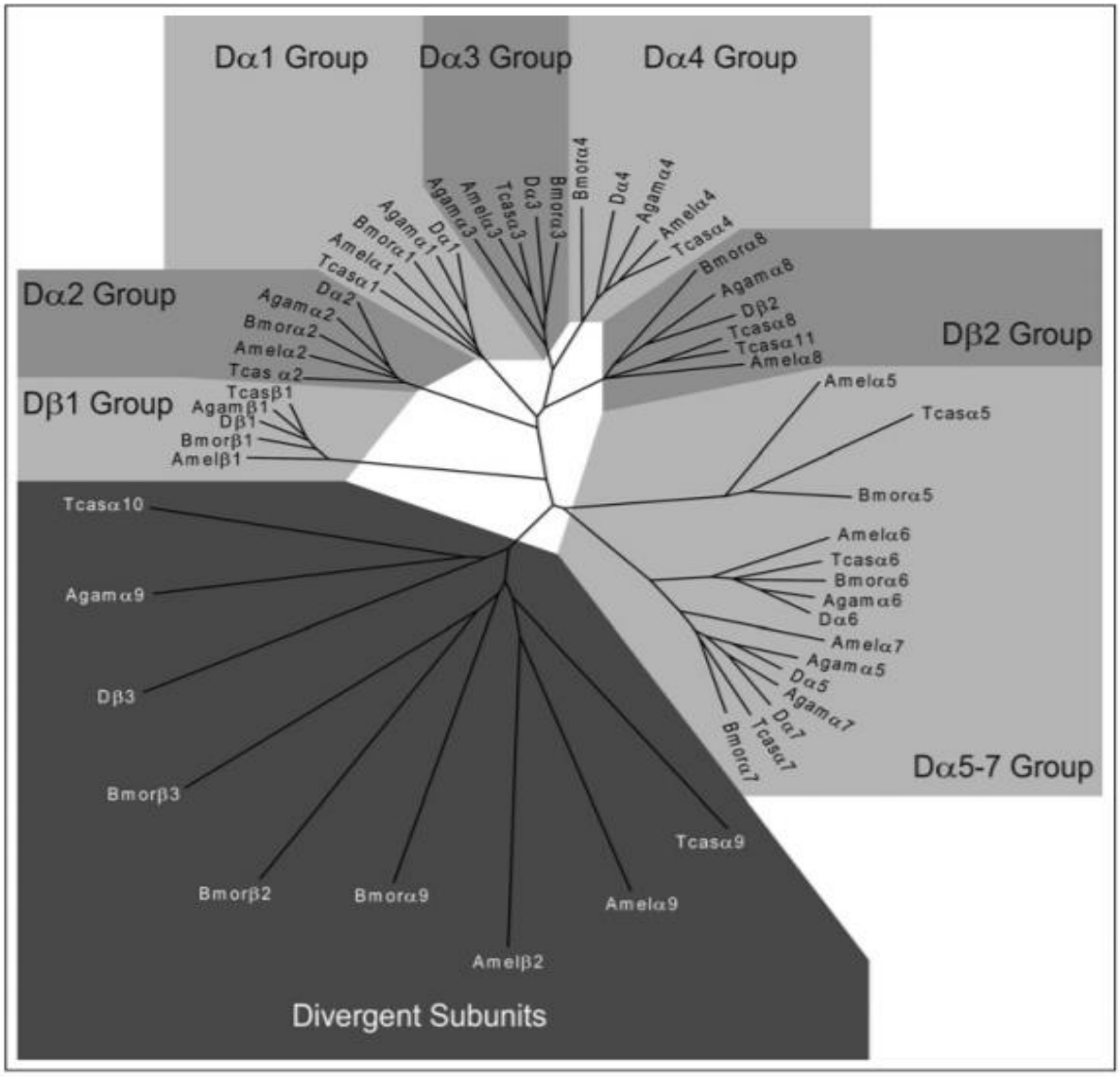

Figure 1.3 Insect nAChR subunits

A map of the nAChR subunits for various insects, divided into 7 groups based on homology with the *Drosophila* subunits e.g. Dα1. All of the subunits within a group contain at least 60% amino acid identity with one another. Additionally shown is the group of divergent subunits; those which are unique to each species. Species can be identified by the shortened nomenclature prefixing the subunit number; Agam is *Anopheles gambiae* (mosquito), Amel is *Apis mellifera* (honeybee), Bmor is *Bombyx mori* (silkmoth) and Tcas is *Tribolium castaneum* (red flour beetle). Figure from Jones and Satelle[72].

1.2.2 Neonicotinoid resistance

One issue with the widespread use of neonicotinoids is that, as pests reproduce significantly faster than many beneficial insects such as bees, they are much faster to develop resistance. For example aphids, a major sap sucking pest which neonicotinoids have been widely used to control[22], can switch between sexual and asexual reproduction[76]. Most aphids are born already pregnant with the next generation, inside which the embryo of the third generation is already developing. Aphids are so prolific that the biologist Thomas Henry Huxley supposedly once calculated that, after ten generations, the progeny on one aphid would have a biomass equivalent to '500,000,000 stout men'[76]. This allows pests like aphids to adapt much more rapidly to insecticide use than bees, who only reproduce once a year. This leads to insecticides having disproportionate effects on non-target species. Pollinators such as bumblebees appear to be at least twice as susceptible to the lethal effects of neonicotinoids than pest species such as aphids[22,35]. Many examples of neonicotinoid resistance in aphids have occurred in the field since use began, with resistance strains being up to ten times less susceptible[34,77,78]. No examples of resistance have been identified in pollinators.

The occurrence of neonicotinoid resistance can provide insights into the mechanism of action and metabolism of neonicotinoids in insects. Resistant strains of pests such as aphids or the brown planthopper appear to have mutations in one of two sets of genes[77-81]. The first set involve mutations in the cytochrome P450 enzyme family. This group of enzymes are responsible for metabolising harmful compounds in insects and appear to be important for the metabolism of neonicotinoids[82,83]. Many resistant strains have some sort of gain of function mutation that leads to overexpression of cytochrome P450s[77-79]. This allows the pests to metabolise neonicotinoids more rapidly, reducing their toxicity. The other common set of mutations observed are those that affect the nAChRs. Loss of function mutations in certain nAChR subunits appear to confer resistance[80,81]. Screening of *Drosophila* strains for neonicotinoid resistance has identified nAChR subunits which seem to play a role in neonicotinoid susceptibility. Loss of function mutations in Dα1 and Dβ2 in *Drosophila* both increase neonicotinoid resistance, suggesting that they are involved in one of or multiple neonicotinoid susceptible nAChRs[84].

1.2.3 Behavioural roles of nicotinic acetylcholine receptor subunits

Alongside neonicotinoid resistance, loss of Dα1 caused a host of behavioural defects, such as disrupted courtship, mating and sleep[85]. This is not the only nAChR subunit that appears to play a role in sleep; a loss of function mutation in *rye,* an nAChR subunit which has high homology with Dα3, causes a reduction and fragmentation of sleep[86]. Other subunits have also been implicated in specific behaviours. For example, Dα7 appears to be concentrated in the dendrites of the giant fibre

system and be involved in startle induced escape behaviour[87]. Mutants with a loss of function in Dα7 showed a lack of escape response. The subunits Dα1 and 4-6 appear to be involved in olfaction, with *RNAi* mediated knock downs of these in the mushroom body output neurons causing a change or even a reversal in response to olfactory stimuli[88]. As mentioned above, Dα1 and Dβ2 are involved in neonicotinoid susceptibility[84], whilst Dα6 is implicated in susceptibility to the pesticide spinosad[89].

Chromosomal loss of function mutations and *RNAi* lines in *Drosophila* have provided many of the insights thus far, and in this thesis *Drosophila* will be further utilised in this way. As discussed, some nAChR subunits appear to play an important role in sleep, including Dα1 which has also been shown to have an important role in susceptibility to neonicotinoids[85]. This suggests that neonicotinoid exposure may disrupt sleep. Sleep is under the control of the circadian clock, which dictates the timing of sleep-wake activity in animals[15]. The clock may represent another unintended target of neonicotinoids as *Drosophila* work has shown that ACh signalling is used to transmit excitatory signals from the light sensing organs to the central clock, and to maintain synchronicity within the clock[90-92]. The effects of neonicotinoids on the clock and sleep have yet to be explored.

1.3 The circadian clock and sleep

1.3.1 The circadian clock

The circadian clock is an endogenous mechanism which allows organisms to time activity and physiological processes with the availability of important environmental factors such as light or food. Due to the evolutionary benefits associated with this, circadian clocks are almost ubiquitous, appearing in organisms from cyanobacteria to mammals[93,94]. The circadian clock results in daily oscillations in gene expression, physiological processes and behavioural activity and is characterised by three main features[95-97]:

1) The rhythm of the clock is entrainable. The rhythm can be set by external cues, called '*zeitgebers*' which translates to 'time-givers'. The most important of these is usually light, but others include temperature, food availability and social cues. These *zeitgebers* set the phase of the clock, which determines where the peak and trough of activity occurs over the 24 hour period.

2) The clock has an endogenous free running period of approximately 24 hours. Circadian rhythmicity is self-sustaining. When environmental cues are removed, and an organism is placed in constant conditions, the clock will continue to run, maintaining circadian outputs such as behavioural rhythmicity with a period of approximately 24 hours. Circadian rhythm assays are often carried out in constant conditions, e.g. constant darkness (DD) to assess this.

3) The clock is temperature-compensated. Most processes in the body speed up as temperatures increases, as this increases the energy available for molecular processes. Enzymatic activity increases with increasing temperature at a rate determined by the temperature coefficient (Q_{10}). However, the clock is able to maintain a 24 hour period across a broad range of climates and despite daily and seasonal changes in temperature.

The clock controls the expression levels of hundreds of genes[95] and the timing of physiological functions leading to daily rhythms in appetite, disease susceptibility and drug efficacy (including insecticides)[98-101]. A disruption to the clock can have profound health effects. In humans, disruption of circadian rhythmicity through shift work, jetlag, stress or chronic pain increases the risk of health issues like depression, obesity, stroke and heart attack[102]. In *Drosophila*, loss of the clock can cause reduced reproductive fitness[103], lifespan and mobility and premature neurodegeneration[96].

1.3.2 Sleep

Sleep is a state of inactivity which is characterised by changes in metabolic rate, posture, arousal threshold and brain activity. Sleep is controlled by two drives, the circadian clock, which dictates the timing of sleep and waking, and the sleep homeostat, which signals sleep need[15,104]. The exact role of sleep is still unknown although it appears to be important for metabolism and synaptic plasticity, aiding in behaviours such as memory[16,105,106]. Given the near ubiquity of behaviours resembling sleep across the phyla, it would appear to be important for survival[107].

1.4 The clock and sleep in *Drosophila*

1.4.1 The circadian clock in *Drosophila*:

One of the best characterised clocks is that of *Drosophila*, who are most active at dawn and dusk[108]. The dominant *zeitgeber* in *Drosophila* is light, however there are other environmental cues that can influence rhythmicity, such as temperature, food availability and social environment. Flies can be entrained by daily temperature changes as low as 4 °C and temperature and light cues can reinforce each other if synchronised[109]. Time of day restricted feeding can influence activity levels and social cues can influence the phase of the clock, with the phase of individuals who've been entrained to different light cycles becoming similar when placed together in constant conditions[110]. This social influence on the clock is olfactory based and dependant on the number of conspecifics. If two differently entrained groups are combined, the larger group with exert a stronger influence over the mixed group's phase[111]. However, if flies are provided with two conflicting cues, light proves to be the dominant *zeitgeber*[109,112-114].

The endogenous clock of *Drosophila* is very strong, with flies who have been maintained in complete darkness for over 330 generations (nineteen years) still showing robust rhythmicity[115]. *Drosophila* has been used to study the circadian clock since at least 1926[116], by tracking the rhythmicity of eclosion, the emergence of the adult from the pupal case. This was one of the methods used to identify *period*, the first clock and behavioural gene to be identified[97]. However, eclosion only occurs once per life time making data collection slow[117]. Most circadian analysis in *Drosophila* now uses locomotor rhythmicity. This allows for the collection of large amounts of continuous data from the same individual, for instance allowing the study of jetlag[118], changing day lengths[119] and aging[120] on the clock.

The first clock gene identified was *period* (*per*), discovered in *Drosophila* by Konopka and Benzer in 1971[97]. After screening multiple mutant lines for circadian abnormalities, they found three lines with abnormal rhythms, all of which had a mutation in the same functional gene, *period*. Eventually, many other clock genes were identified, including *timeless* (*tim*)[121], *clock* (*clk*)[122] and *cycle* (*cyc*)[123]. This allowed the transcription feedback loops at the very core of the *Drosophila* molecular clock to be identified. This feedback loop has proven to be a principle mechanism of the circadian clock, with the mammalian molecular clock possessing homologues of most of these genes and functioning in a very similar way. The work that led to deciphering the mechanism of the clock using *Drosophila* was recognised when Jeffery Hall, Michael Rosbash and Michael Young and were awarded the 2017 Nobel prize for Medicine or Physiology 'for their discoveries of molecular mechanisms controlling the circadian rhythm'.

1.4.2 The molecular clock

In *Drosophila*, the *per-tim* transcription loop forms the core of the molecular clock (Fig. 1.4). Both *per* and *tim* are transcribed during the day, leading to the build-up of *per* and *tim* mRNA in the nucleus, peaking in early evening[124]. At this point, translation of PER and TIM proteins occurs and they accumulate in the cytoplasm. Controlled phosphorylation of TIM and PER allows formation of a stable heterodimer which is then able to undergo entry into the nucleus in the middle of the night[125]. The presence of PER:TIM in the nucleus prevents the transcription of *per* and *tim*, through inhibition of their transcription activation factors CLK and CYC[126]. In the morning, levels of TIM and PER decrease and *per* and *tim* mRNA levels begin accumulating again. Thus, this autoregulatory, negative- feedback loop allows daily oscillations in clock gene expression.

Clock and *cycle* also form another, secondary transcription loop. The CLK:CYC complex drives expression of *per* and *tim*, through binding to the E box of the promotor region, but also of PDP1 and, in turn, VRILLE1, which represses the expression of *clk*. It is thought that this second loop

increases the accuracy and stability of the first[127,128]. The occurrence of E box regions in genes other than *per* and *tim* allows the clock to cause oscillating expression of many other output genes, through CLK:CYC promoted expression, leading to downstream circadian outputs and changes in physiology[129,130].

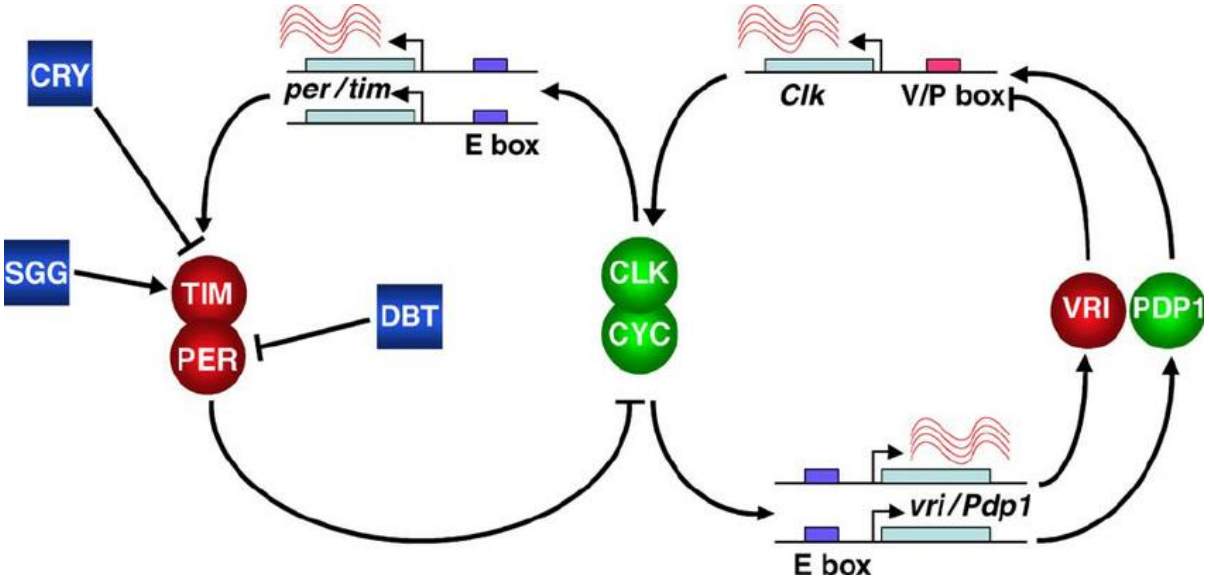

Figure 1.4 The core molecular clock in *Drosophila*

An illustration of the key components of the molecular clock in *Drosophila*. The genes *period* (*per*) and *timeless* (*tim*) are expressed, leading to an accumulation of PER and TIM in the cytoplasm. These form a complex which is then transported into the nucleus and prevents further transcription of *per* and *tim* through inhibition of Clock (CLK) and Cycle (CYC). The rate of this transcription loop is roughly 24hrs and is set by various phosphorylation and dephosphorylation factors such as Doubletime (DBT), Casein Kinase II (CKII), Shaggy (SGG) and Protein Phosphotase 1 (PP1) 2A (PP2A). Photosensitive CRY allows the degradation of TIM, leading to a light induced resetting of the clock. Figure adapted from Collins & Blau[131].

The 24 hour periodicity of the molecular clock is reliant on a number of critical delays, to control the timing of protein accumulation in the cytoplasm and of transportation of PER:TIM into the nucleus. This timing is controlled largely through the presence of various phosphorylation and dephosphorylation factors. For example, dephosphorylation of PER by Protein Phosphatase 1 (PP1) or 2A (PP2A) stabilise the protein[132-134], whist phosphorylation by doubletime (DBT) marks it for degradation[135]. Phosphorylation by Casein Kinase II (GSKbeta3) and shaggy (SGG) promote the entrance of PER:TIM into the nucleus[136,137]. These processes are important for controlling the periodicity of the clock and null mutations in these factors or mutations in the phosphorylation sites of PER can cause lengthening or shortening of the period length[133-138].

The molecular clock is set by the blue light photosensitive cryptochrome (CRY). CRY allows the light dependant degradation of TIM[139]. Without TIM, PER is destabilised and also degrades, leading to a light induced resetting of the clock and preventing PER and TIM protein levels from accumulating until the evening[124]. Light can penetrate the cuticle, allowing light cues to set the pace of clock neurons in the brain directly.

1.4.3 The clock neurons

The central clock in the fly consists of a few circadian gene expressing neurons in the brain. In total, there are approximately 75 pairs of symmetrically arranged clock neurons, divided into seven groups based on their anatomical position in the brain[124,140] (Fig. 1.5). These groups differ in their expression of genes, neuropeptides, neurotransmitters and receptors and have different functions within the clock. The lateral neurons, consisting of the lateral posterior neurons, (LPNs), the dorsolateral neurons (LNds) and the small and large ventrolateral neurons (s-LNvs and l-LNvs) appear to be most involved in the entrainment of the clock and contain a large number of *cry* expressing cells[112]. The three groups of dorsal neurons, DN1s, 2s and 3s appear to carry out modulation of the clock, including integration of temperature cues and influencing the phase of rhythmicity in constant conditions. Few of the DNs express *cry* and they seem to entrain more readily to temperature cycles than light cycles[112].

The LNvs make up the key pacemaker of the clock. These cells, except the 5th s-LNv, express the neuropeptide pigment dispersing factor (PDF)[141]. This neuropeptide is necessary for circadian behavioural rhythmicity and important for communication amongst the clock neurons[142,143]. PDF receptor (PDFR) expression occurs in approximately half of the clock neurons throughout the clock neuron network and even in the LNvs themselves[144]. PDF signalling is important for the organisation of the clock, allowing the pacemaker cells to influence the rhythmicity of the other, downstream clock neurons. PDF signalling is not required for the function of the molecular clock. However, loss of PDF signalling causes the pacemaker cells to become asynchronous, as well as resulting in the other clocks neurons falling out of phase with one another[143]. It is thought that the s-LNv dorsal terminals are important for this communication and synchronisation of downstream clock neurons. The s-LNv dorsal terminals make contact with the DN1 dorsal clock neurons and show circadian plasticity in arborisation and oscillations in PDF accumulation[145]. The terminals are significantly more branched and have greater PDF accumulation in day time than at night time[146]. This remodelling and cycling in PDF accumulation does not occur in canonical clock mutants, such as *per*[147].

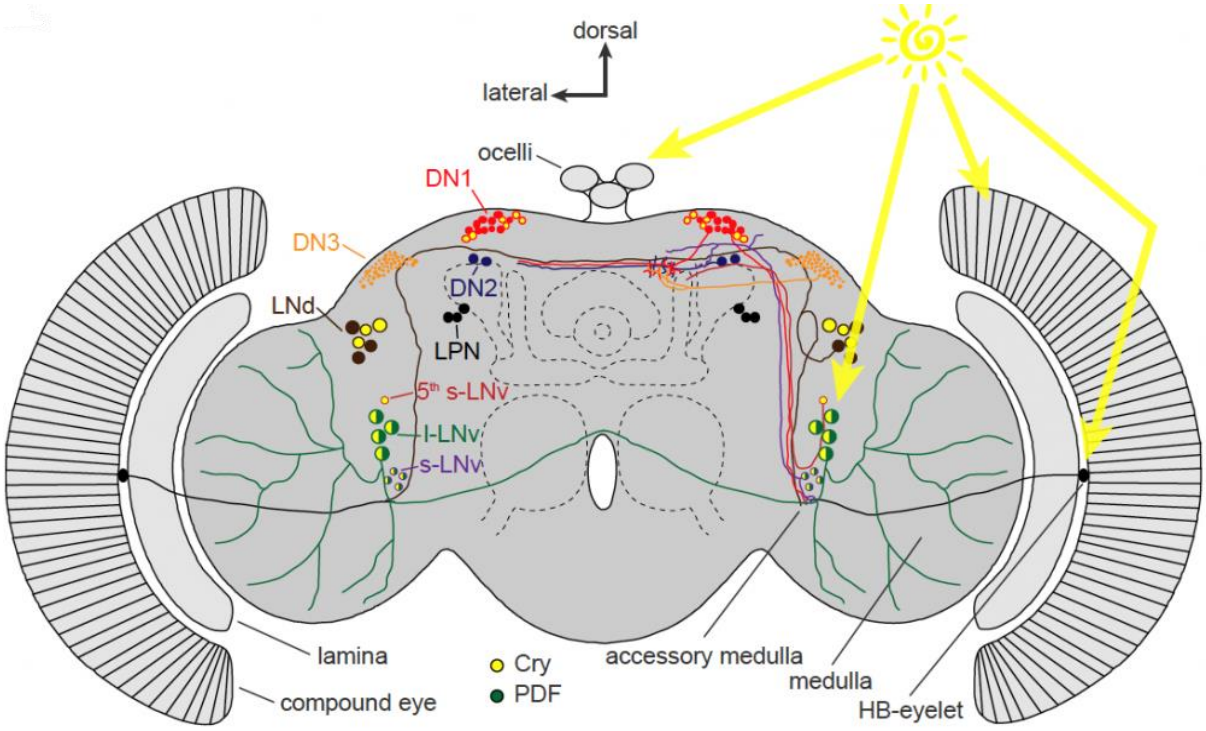

Figure 1.5 The central clock in *Drosophila*

A depiction of the clock neurons in *Drosophila*, showing the three dorsal groups (DN1-3 and) and the four lateral groups (s-LNvs, l-LNvs, LNds and the LPNs). In yellow, the clock neurons which express *cryptochrome* (*cry*) and in green, the neurons which express PDF. As shown, light can enter the system via *cry* or through the light sensing organs (the ocelli, compound eyes and the Hofbauer-Buchner (HB) eyelet). Figure from Buhl et al[148].

The s-LNvs maintain the clock during the night, providing the morning peak in activity and delaying the evening peak[149,150]. They are also responsible for rhythmic behaviour in constant conditions, maintaining a robust molecular clock in the absence of light[151]. The l-LNvs are important for light-mediated arousal under a light cycle of 12 light, 12 hours dark (LD)[152,153], but cannot maintain rhythmicity in continuous darkness (DD)[154]. However, they are important for maintaining synchronicity between the two hemispheres, *via* projections to the contralateral LNvs through the posterior optic tract[92]. They also appear to synapse to the s-LNvs, likely enforcing their rhythmicity in LD[155] as well as signalling to the LNds[156], which are responsible for the evening peak in activity[149,150]. The presence of two coupled oscillators, one which drives the morning activity peak (s-LNvs) and one which drives the evening peak (LNds) allows the clock to adjust to seasonal changes in day length[157].

Both the s-LNvs and the l-LNvs appear to make direct contact with light sensing organs, providing another route for light to enter and entrain the central clock. Both sets of LNvs receive excitatory ACh signalling[90,91]. In the l-LNvs this comes from the visual circuitry such as the lamina, whilst in the s-LNvs it appears to come from the Hofbauer-Buchner eyelet (HB eyelet)[90,91,156]. Both cry and signalling from the light sensing organs are sufficient to entrain the clock on their own although the loss of either weakens behavioural rhythmicity[158].

The central clock produces circadian outputs in a number of ways. One way is through direct synaptic connection to output circuits. For example, the DN1s, which are synaptic partners directly downstream of the s-LNvs, are also important for the morning activity peak and form part of the output pathway responsible for behavioural rhythmicity. The DN1s provide a link between the s-LNvs and non-clock cells in the *pars intercerebralis* (PI) which are required for rhythmic activity[159]. Another way that the central clock can produce circadian outputs is through setting the pace of peripheral clocks in other tissues and cells, allowing central synchronisation of physiological events throughout the animal. One example of this is the oenocyte cells. These cells produce pheromones and have clocks which can be set by PDF from the CNS, which is thought to reach them *via* the circulating haemolymph. Disruption of PDF signalling in *Drosophila* causes a reduction in sex pheromone production, causing a change in mating timing and frequency[13].

Although some peripheral clocks, such as those in the oenocytes, appear to be controlled by the central clock, many peripheral clocks are autonomous. These autonomous clocks are set by environmental cues. For example, the clocks in the malpighian tubules, which form part of the excretory system in flies, contain *cry*[160], are set by light and their phase is not influenced by the central clock. Malpighian tubules that are transplanted from a donor fly into a host fly entrained to conflicting light dark cycles will maintain the phase of the donor fly[161].

1.4.4 The membrane clock

The electrical activity of the clock neurons and the day/night differences in their electrical state is referred to as the 'membrane clock' and is integral to the production of circadian outputs. The molecular clock is best characterised in the l-LNvs, as their greater size makes electrophysiology more feasible. Whole cell patch clamp of these cells over the 24 hour period found daily oscillations in the resting membrane potential (RMP), spontaneous action potential (AP) firing rate and input resistance[162]. In the l-LNvs, the RMP, AP firing rate and input resistance were all greater in the day than at night and all peaked at lights on, leading to greater excitability in the l-LNvs during the day than at night. This same pattern, with electrical excitability peaking at dawn, is also observed in the s-LNvs and a subset of DN1s, which fits with their role as 'morning cells' and the morning peak in

behavioural activity[162,163]. In the l-LNvs, this oscillation in excitability is abolished in *per*^{01} null mutant flies or by placing wildtype flies in DD, suggesting that oscillation of clock genes is required for the oscillation in excitability in the clock neurons[162]. The mechanism for this is not completely understood but clock genes promote the cyclical expression of membrane ion channels in the pacemaker neurons, such as Ir, an inwardly rectifying K^+ channel[164]. It is likely that through promotion of daily oscillations in multiple membrane ion channels and receptors, the molecular clock can cause day/night differences in membrane excitability[163].

The membrane clock is necessary for many circadian outputs and acts to enforce and maintain the molecular clock. Hyperexcitation of the LNvs causes PDF cycling in the dorsal terminals of the s-LNvs to cease, causing an accumulation of high levels of PDF[165] . This suggests that day/night differences in membrane potential are necessary for the synthesis, transport or release of PDF from the s-LNvs, although the specifics of this process are unknown. Potentially due to this change in PDF cycling, hyperexcitation also causes desynchronisation of downstream clock neurons, causing breakdown of behaviour into complex rhythms and phase shifting of the DN1 and DN2 neurons[165]. Similar results have been observed for flies that contain clock neurons expressing K^+ channels that result in electrical inactivation of the l-LNvs. These flies exhibit disrupted behavioural rhythmicity and changes in the phase of downstream clock neurons *e.g.* the LNd, DN1 and DN2 neurons[166]. PDF cycling in the s-LNv dorsal terminals ceases as does circadian remodelling, with terminals having low arborisation and low PDF accumulation in the daytime[151]. When put into DD, flies with electrically-silenced LNvs show a rundown of the molecular clock, suggesting that electrical feedback is also required for the maintenance of the circadian clock under constant conditions[167].

1.4.5 Sleep in *Drosophila*

The circadian clock and sleep are interlinked. As the clock controls the timing of activity, so too does it control the timing of inactivity, or sleep. *Drosophila* sleep throughout the night and show a siesta at midday. In *Drosophila*, sleep is defined as inactivity lasting longer than five minutes[168]. Although there is some debate as to whether flies truly sleep or not[169], these bouts of inactivity do appear to coincide with other physiological markers associated with sleep in other animals. For example, the arousal threshold of flies increases, meaning that a more intense stimuli is required to elicit movement from them[168]. If sleep-deprived, flies show rebound sleep the following day, showing that sleep is under homeostatic control[170]. Also, they show reduced neuronal activity and reduced neuronal responsiveness to sensory stimuli during sleep[171]. Flies respond to stimulants such as caffeine and sedatives such benzodiazepine in the same way as humans and other animals[172]. Like mammals, flies also appear to cycle between shallow and deep sleep, and to achieve deeper sleep

during the night[168,173]. This sleep phase is important for memory consolidation. Sleep deprivation in adults can cause learning and memory deficits whilst inducing sleep can increase learning capacity[105,174]. Flies have longer or more intense sleep after learning tasks[175,176]. Although learning deficits can be undone by allowing flies to sleep, if sleep deprivation occurs during development then there can be long lasting memory defects and disruption to behaviours such as courtship, potentially due to impaired development of one of the olfactory glomeruli, VA1v[177,178]. This shows how important sleep is to the development and adult plasticity of the brain. Sleep is also hypothesised to be important for synaptic homeostasis, allowing down-regulation of the strength of synaptic connections[179]. This is thought to improve the signal-to-noise ratio and reduce energy expenditure in the brain[180]. Sleep deprivation appears to reduce cognitive performance, reducing both the consistency and amplitude of the neuronal response of flies to sensory stimuli[171].

There are multiple groups of neurons in various brain regions which have been implicated in sleep. The l-LNvs appear to be the main connection between the clock and sleep and are the key arousal neurons in the sleep-wake circuity[181]. This role appears to involve signalling from the visual circuit, allowing arousal in light conditions and sleep during dark conditions. Flies with hyperexcited l-LNvs sleep less and their l-LNvs do not respond electrically to light input[153].

Another brain region which has been shown to influence sleep are the mushroom bodies and their output neurons. The mushroom bodies are a pair of structures in the brain that receive olfactory input and are involved in learning and memory in insects[182]. They also appear to contain both sleep and wake promoting neurons[183,184]. In the mushroom body output neurons, these seem to be divided by neurotransmitter usage, with the two identified wake-promoting groups being glutamatergic and the two sleep promoting groups being cholinergic or GABAergic[185]. There are also sleep promoting neurons in the dorsal fan shaped body, namely the lateral extrinsic fan-shaped body (EXFl2) neurons. These can be inhibited by dopamine, giving dopamine a wake-promoting role in the brain, like in mammals[105,186]. The neurotransmitter octopamine also appears to play a wake promoting role, for example the anterior superior medial neurons that project to the *pars intercerebralis,* promote wakefulness when activated through octopamine signalling[187]. There are also a group of wake-promoting DN1s which project to the *pars intercerebralis*. These are the neurons which were previously mentioned as appearing downstream of the s-LNvs and promoting rhythmic locomotor activity[159].There is also a small group of wake-promoting neurons in the *pars lateralis*[188].

The dopaminergic, EXFl2 neurons in the dorsal fan shaped body may also play a role in the sleep homeostat, as their electrical activity increases with increasing sleep deprivation[189]. The R2 neurons

of the ellipsoid body (EB) also seem to be an important part of the sleep homeostat circuitry. These neurons fire more the longer the animal has been awake, and show an increase in synapse number and size. Activation of these neurons, even for a short period of time, can cause increased sleep for the next twelve hours[190,191]. A small group of DN1's project to these EB R2 neurons, seeming to connect the circadian clock and the sleep homeostat. Activation of these DN1's induces sleep like oscillations in the EB R2 neurons and increases the arousal threshold[192]. The DN1's also form synapses onto the s-LNvs and LNds, using glutamate to inhibit them to allow sleep during the night[193]. This signalling is increased by increasing temperatures, providing a pathway for temperature to promote sleep. Sleep can also be influenced by other environmental factors, for example starvation reduces sleep[194], whilst social enrichment leads to increased sleep[175].

1.5 The clock and sleep in the bee

1.5.1 Bee circadian clock

The circadian clock is integral to foraging efficiency in pollinators. Many aspects of floral resource availability, such as flower opening, scent release and nectar production are time of day dependant[11]. This has led to foragers possessing an impressive ability to encode information and communication temporally[195]. Honeybee foragers are able to learn to forage accurately at multiple feeders which are rewarding at different times of day[196], as little as twenty minutes apart[197]. The time of day is used along with other features such as colour and odour to create a '*gestalt*'; a robust memory of the rewarding resource. The time of reward is an integral part of this *gestalt*; if it is not reliable, learning success decreases by 9%[198]. The circadian rhythm also directly affects their ability to learn; honeybees learn novel, rewarding odours better in the morning[199]. This is thought to help them find new foraging patches, as most flowers are nectar-rich in the morning[11].

This circadian time keeping also allows honeybees to communicate resource availability through the waggle dance. The waggle dance uses the sun's azimuth as a landmark, requiring the dancer to know the position of the sun while dancing inside the darkness of the colony[12]. Some 'marathon dancers' continue to dance for hours, accurately shifting the angle of their dance to match that of the moving sun. Honeybees also experience 'jetlag'; if moved between time zones foragers initially miscommunicate, using the azimuth from the last time zone rather than the current one[200].

The bee clock can be entrained by a number of different environmental cues. Like flies, honeybees and bumblebee foragers entrain very accurately to light cycles[201,202]. Honeybees are also capable of entraining to temperature cycles of 10 °C, while lower fluctuations can alter or enforce entrainment to LD cycles[203]. Unlike flies, honeybees also show robust entrainment to food availability in constant

conditions[204] and restricted feeding can change the phase of the molecular clock in LD conditions[205]. The social environment also has a big influence on the clock. The foragers of a honeybee colony placed into constant conditions will stay in phase with one another, whereas foragers who are isolated from one another quickly fall out of phase[201]. If two groups of foragers are entrained to opposing phases and then integrated in DD, the foragers will rapidly fall into a synchronous phase halfway between the two entrained phases[206]. Young worker bees need to be exposed to the colony environment or rhythmic conspecifics to develop rhythmicity[207]. In fact, unlike in flies, the social environment appears to be a stronger zeitgeber than light, with foragers exposed to opposing light and colony phases aligning with the colony rather than the light cues[208].

The presence of the queen also appears to influence forager rhythmicity. Although the honeybee queen herself is arrhythmic, if kept in an LD cycle 8 hours ahead of a group of foragers and then introduced into the group, she shifts their cycles 1.38 hours forward[209]. This suggests that the queen contributes to colony synchronicity, perhaps through circadian pheromone release.

1.5.2 Task related plasticity in the bee clock

The task dependent partitioning of circadian rhythms that allows the occurrence of an arrhythmic queen and rhythmic foragers is a peculiarity of eusocial species. In honeybee and bumblebee colonies, the queens and in-nest workers are arrhythmic, as tasks such as brood care must be carried out round the clock[210,211]. However, foragers exhibit strong circadian rhythmicity. Bees are able to transition between arrhythmic and rhythmic behaviour as they transition between different roles in the nest. For example, in honeybee colonies, tasks are partitioned by age, with workers carrying out in-nest work initially and then transitioning to foraging as they age, developing rhythmic behaviour concurrently. This switch to rhythmicity is not irreversible; if a generation of nurses is removed from the honeybee colony foragers are capable of switching back to this task and the associated behavioural arrhythmicity[212]. A similar phenomenon occurs in bumblebee colonies, although there, task partitioning occurs by size rather than age, reducing the degree of task flexibility[19]. In bumblebee colonies, the queen also exhibits circadian flexibility, as she is required to initiate the colony, foraging rhythmically to provide for the first generation of workers[213]. Once these workers begin foraging, the queen remains within the nest and reverts to arrhythmicity. It appears that the attenuation of rhythmicity seen in nest workers may occur *via* some sort of olfactory cue from the brood, although the mechanism for this has yet to be elucidated[210]. Despite their behavioural arrhythmicity, nurse honeybees show oscillation in PER levels in the brain that peak at the same time of day as in foragers[214]. When removed from the colony and isolated in constant conditions, nurse honeybees and bumblebees develop circadian rhythms which are synchronised with the rest of the

colony[211,215]. This suggests that there are a subset of clocks still oscillating in the nurse bee which are able to set the pace of the clock once they are removed from a brood related signal which causes the decoupling of behavioural rhythmicity from the clock[216,217].

1.5.3 The molecular clock in bees

The molecular clock in honeybees resembles that of *Drosophila*, with orthologues for the putative clock genes *per, cry*, *clk* and *cyc*[218] (Fig. 1.6). The *cry* found in bees is homologous to the mammalian *cry2*, rather than the *cry* found in *Drosophila* (*cry1*) meaning that it is not light sensitive[218]. In bees, *cry* appears to have replaced *tim* in the *per:tim* feedback loop, with *tim* having been lost. Otherwise, the principles of the feedback loop remain the same, with CRY and PER forming a complex which enters the nucleus, inhibiting CLK and CYC and preventing them from promoting further expression of *cry* and *per*[218]. The second feedback loop observed in *Drosophila* also appears to occur in bees, with homologues of VRILLE and PPD1 occurring which have 94-100% amino acid identity with *Drosophila* for the *clk*/*cyc* binding regions[218].

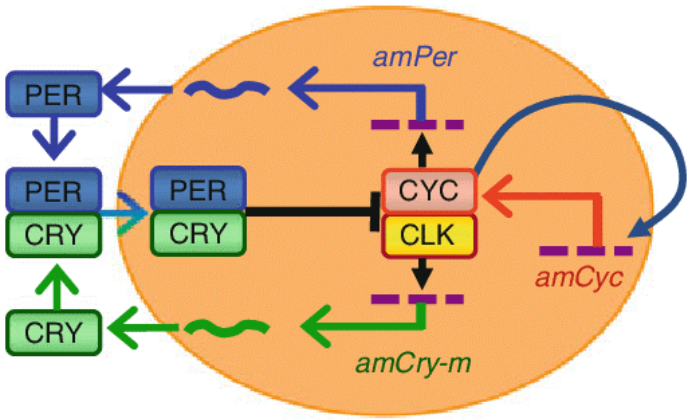

Figure 1.6 The molecular clock of the honeybee

A depiction of the core molecular clock in the honeybee. The genes *per* and *cry* are expressed in the nucleus (shown in orange), leading to accumulation of PER and CRY in the cytoplasm. These then form a dimer, which enters the nucleus and prevents further transcription of *per* and *cry* by inhibiting their transcription factors CLK and CYC. Figure from Bloch[219].

It is unclear precisely how light signals are integrated into the molecular clock in the absence of a light-sensitive CRY. Potentially *tim2,* which is expressed in the retinas in mammals*,* plays some part[220]. *Tim2* is a homologue of *timeless* is *Drosophila*, in which it has been shown to participate in light entrainment[221]. In honeybees, *tim2* oscillates in both constant dark (DD) and constant light (LL) but with a different phase, so may interact with both light and the clock[218]. Alternatively, light may enter the system through non-visual opsins such as pteropsins. Pteropsins are a group of opsins which are expressed in the brain but not the eyes or ocelli of honeybees[222]. They appear to be homologous to a c-opsin that has recently been found in platynereis and shown to be a route *via* which light entered the circadian clock[223].

Oscillation of the clock genes influences the expression patterns of many other genes in the bee. For example, both nurse bees and foragers show circadian oscillations in the expression of P450 enzymes, suggesting that susceptibility to neonicotinoids may vary throughout the day[224].

1.5.4 The bee clock neurons

Although the central clock of the bee has not been as well characterised as in *Drosophila*, the basic structure seems to be similar. In honeybees there are four bundles of neurons, two lateral and two dorsal, which express *per*[214] (Fig. 1.7). One of the lateral groups, the lateral neurons group 2 (LN2's) consist of approximately 15 neurons per hemisphere and express both PER and PDF, suggesting that they may be the key pacemaker cells in bees[225]. These neurons project widely throughout the brain. The PDF accumulation in these cells oscillates throughout the day, increasing through the day and decreasing during the night. This cycling of PDF is observed in both foragers and nurses. The PDF expressing/positive (PDF+) neurons come into close proximity with the other PER+ neurons and injection of PDF into the brain of honeybees can cause a significant phase delay. This suggests that, like in *Drosophila*, PDF may be important for communication and rhythmicity in the bee clock[225]. The number and location of PDF+ neurons in *B. terrestris* matches that of honeybees[226].

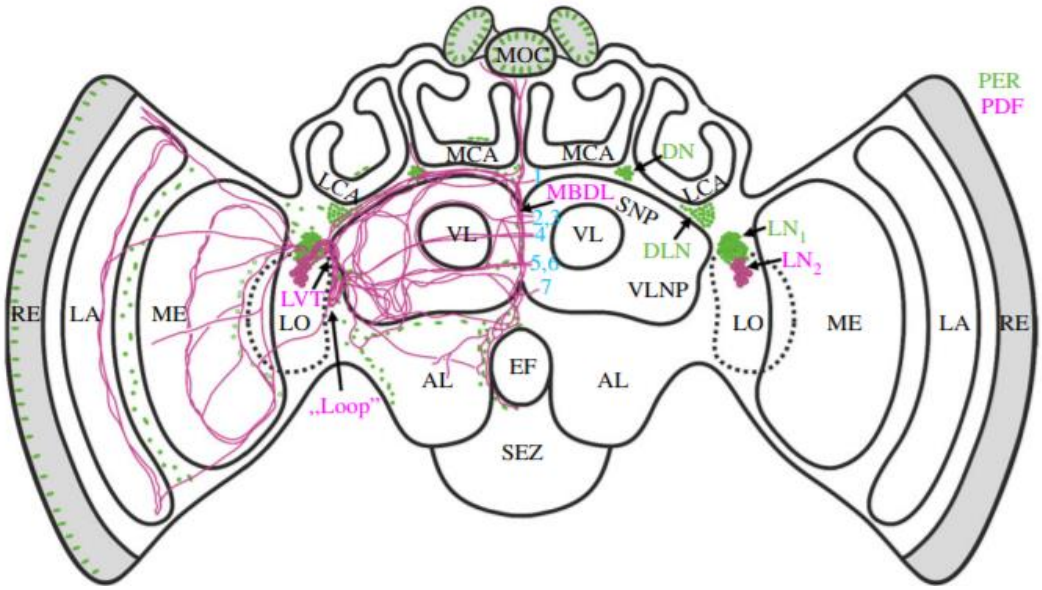

Figure 1.7 The central clock in the honeybee

The clock neurons of the honeybee brain. In green, the 4 bundles of neurons shown to express PER and in pink, the bundle of neurons which express both PER and PDF. On the left, the extensive projections of these PDF+ neurons are shown, with projections towards the areas such as the light sensing organs and the mushroom bodies. Figure from Beer et al[225].

1.5.5 Sleep in the bee

The honeybee was one of the first invertebrates in which sleep was identified[227]. Sleep in bees shares many characteristics with mammalian sleep. In honeybees sleep corresponds with relaxation of the muscles, a reduction in metabolic rate and body temperature and an increase in arousal threshold[228]. They also show sleep specific antennal movements which occur towards the end of sleep episodes, during deep sleep, and have been compared to rapid eye movement (REM) in humans[228]. Like in *Drosophila*, sleep in the bee is important for memory performance and consolidation. In honeybees who have undergone appetitive training, presentation of the training odour during deep sleep increases the strength of the memory[106]. Sleep deprivation reduces the ability of foragers to remember a new navigational route and reduces the accuracy of the angle, the temporally encoded aspect, of the waggle dance[16,229]. This implies that sleep deprivation reduces forager's ability to accurately consult the clock. Sleep deprivation also results in rebound sleep the following night[230].

1.5.6 Potential routes for neonicotinoid induced disruption of the clock and sleep

As has been laid out, the extensive work in *Drosophila* suggests that ACh signalling is an important component of both the circadian clock and the sleep wake cycle. The PDF expressing LNvs of the fly clock are excited by ACh, expressed by the light sensing organs[90,91]. The l-LNvs also utilise ACh signalling to maintain synchronicity between the two hemispheres and are also vital arousal neurons in the sleep-wake circuitry[92,153]. A subset of another group of clock neurons, the LNds also appear to use ACh signalling to communicate with the s-LNvs[231]. The electrical state of the LNvs is under circadian control and is important for the production of circadian outputs, suggesting that interference of these excitatory pathways by neonicotinoids could cause largescale disruptions to rhythmicity and sleep. Additionally, the sleep promoting subset of the mushroom body output neurons and the Kenyon cells in the mushroom bodies are cholinergic, providing another route by which sleep may be affected[88, 185, 232].

1.6 *Drosophila* as a model

Drosophila represents an important potential tool for the collection of evidence of the harmful effects of insecticides on beneficial insects. *Drosophila* are a well-established model organism which has been used for modelling disease, drugs and neuroanatomy for decades[233-235]. *Drosophila* have a relatively simple genome and neuroanatomy, for example their brains contain just ~135,000 neurons, making them comparatively easy to work with. Despite this they can be successfully used to model a range of complex behaviours, including those associated with eusociality, for example the effect of the queen mandibular pheromone on worker ovary maturation[236]. Additionally, *Drosophila* are small, with a body mass approximately 100x less than that of *B. terrestris*[237,238], have a shorter developmental period (10 days compared to 5 weeks) and are very cheap to keep and assay at high quantities. *Drosophila* has been used as the insect model organism for over 100 years and is both highly genetically tractable and has a large number of well-established, reliable assays and reagents for testing behaviour, for example the *Drosophila* Activity Monitor (DAM) setup for assessing circadian rhythmicity and sleep which will be introduced in detail in Chapter 2.

The main benefit of using *Drosophila* is their genetic tractability. The fly genome has been sequenced since 2000[239] and there are many tools for manipulating gene expression, *i.e.* over expression or knockdown of endogenous genes or expression of transgenes, allowing exploration of their role. Two tools which are used with great frequency are RNA interference *(RNAi)* lines and the *GAL4-UAS* system[240,241]. These can be used in conjunction to allow the silencing of genes in a tissue-specific way, allowing one to determine where and when a gene functions *in vivo*. In flies, *RNAi* lines express double-stranded RNA (*dsRNA*) which is complimentary to the endogenous target gene. This

dsRNA is cleaved by an enzyme called dicer into two single strands, the guide strand then binds to the complimentary endogenous *mRNA*, inducing cleavage and resulting in post-transcriptional silencing of the target gene.

This can be combined with the *GAL4-UAS* system to allow tissue specific gene silencing. The *GAL4-UAS* system comprises two parts, the upstream activator sequence (*UAS*) and the yeast transcription activator protein *GAL4* (Fig. 1.8). When the *GAL4* transcription factor binds to *UAS*, it causes transcription of the gene downstream of the *UAS*. As there are no naturally occurring *UAS* regions in *Drosophila* this allows very targeted gene expression. Libraries of *GAL4* lines have been created with *GAL4* expression in specific tissues, brain regions or even a small number of neurons. When these are crossed with *UAS*-lines where the *UAS* region is next to a specific target gene, flies are created with target gene expression localised to a specific tissue or region. In the case of *UAS-RNAi* lines, this allows the knock-down of a single gene in a single region of the fly or fly brain. Other commonly used genetic tools include loss of function mutations, gene overexpression, overexpression of transgenes e.g. human disease genes and CRISPR-CAS9 technology to make knock-out or knock-ins of genes with higher efficacy.

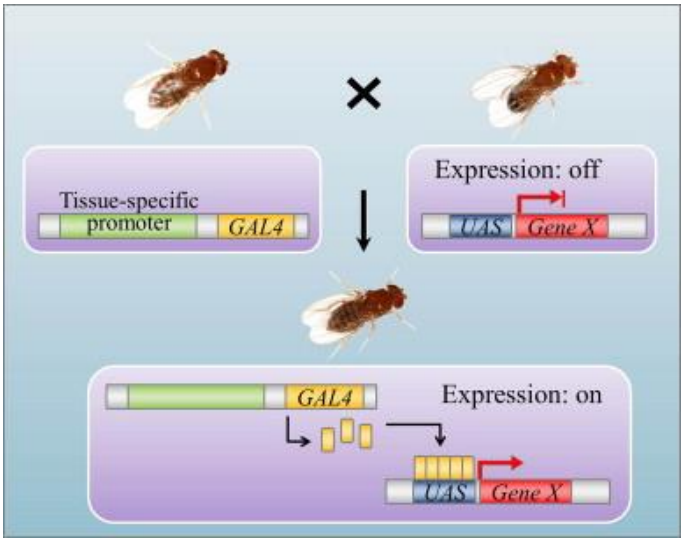

Figure 1.8- The *GAL4-UAS* binary transgenic expression system in *Drosophila*

A depiction of the *GAL4-UAS* system, that allows targeted gene expression in *Drosophila*. One parent line contains the *GAL4*, expressed in a specific tissue, whilst the other parent line contains the *UAS* promotor region, located next to the gene of choice. When crossed this produces offspring with the desired target gene expression pattern. *GAL4* and *UAS* are not normally expressed in flies, therefore *GAL4/+* and *UAS/+* should be wildtype. Figure from Cho et al[242].

Previous work has already shown that *Drosophila* are an apt model for exploring both the insect clock and neonicotinoid exposure. *Drosophila* has already been used to model the clock in bees. Fu & Whitfield[243] identified ten genes whose expression changed in honeybee brains when they switched from arrhythmic nest work to rhythmic foraging. *Drosophila* mutants with *RNAi* knock downs of these ten genes exposed six which are involved in locomotor rhythmicity, shedding new light on the mechanism behind circadian plasticity in bees[243]. This shows how the genetically tractable *Drosophila* can be a valuable tool when exploring behaviour in other, less well characterised insects, with perhaps greater ecological importance. *Drosophila* have also been utilised to explore neonicotinoid resistance, through the creation and screening of loss of function mutants for specific nAChR subunits[84]. This has provided insight into the possible conformation of neonicotinoid susceptible nAChRs. However, there is still a lot of untapped potential for the use of *Drosophila* as a model insect for insecticide effects and their modes of action, aided by the homology of nAChRs between insects. In addition to being an excellent model, *Drosophila* also fill both pest and pollinator niches in the wild, e.g. *Drosophila suzukii* is a pest of soft fruit which has recently expanded its range into Europe[244] whilst *Drosophila hydei* is an important pollinator of orchids[245], providing further value to exploration of insecticides in these insects.

1.7 Thesis aims and structure

The key aim of this thesis is to identify the effects of neonicotinoid pesticides on the insect clock and sleep. *Drosophila* is used as a model, to extensively characterise the effects of neonicotinoids and to attempt to identify where in the brain the neonicotinoids are acting and *via* which nAChR subunits. The effects on rhythmicity and sleep are then also tested on foragers of the bumblebee *B. terrestris*.

Chapter 2 details the materials and methods used throughout this thesis. Chapter 3 describes and discusses the effects of four neonicotinoids on the behavioural rhythmicity and sleep of *Drosophila*. Chapter 4 identifies nAChR subunits which seem to be involved in the effect of neonicotinoids on the clock and sleep and illustrates the effect of neonicotinoid exposure on clock neuron plasticity. Chapter 5 shows the effects of neonicotinoid exposure on sleep and locomotor rhythmicity in isolated bumblebee foragers and on foraging rhythmicity in the full colony setting. Chapter 6 discusses the findings of this thesis and summarises the potential implications for insects experiencing neonicotinoid exposure in the field. The appendix contains a manuscript for a paper detailing the effects of neonicotinoid exposure on the clock, learning and memory and other sub-lethal effects in *Drosophila*.

2

Chapter 2: Materials and Methods

This chapter will provide an overview of the materials and methodology used in this thesis. Section 2.1-2 summarises the fly and bee stocks used and how they were maintained. Section 2.3 covers the climbing assay, 2.4-2.5 detail the circadian rhythmicity and sleep assays in *Drosophila*, while 2.6-2.7 explain the circadian rhythmicity and sleep assays for *B. terrestris* foragers. Section 2.8 outlines the immunohistochemistry work for quantifying the branching and PDF accumulation at the s-LNv dorsal terminals and 2.9 covers the statistical analyses carried out.

2.1 Fly stocks

Flies were reared in plastic bottles (60mm x 130mm, SLS, # FLY1012) or vials (25mm x 95 mm, SLS, # FLY1102) stoppered with cellulose acetate Flugs® (SLS #49-103) and containing approximately 50ml (bottle) or 10ml (vial) of fresh fly food. Food was made up in quantities of 5L and consisted of 400g polenta, 35g granulated agar, 90g active dried yeast, 50g soya flour, 400ml malt extract and 200ml molasses, with 40 ml of propionic acid (Sigma-Aldrich, #94425) and 100 ml of nipagin (Sigma-Aldrich, #H5501) added once cool. The addition of propionic acid makes the food attractive for egg-laying and nipagin acts as an anti-fungal agent[246,247]. Where neonicotinoids were added, this was done after the food had cooled, from a frozen and aliquoted stock solution of 100,000 µg/L ddH_20. The neonicotinoids used were all analytical standard (PESTANAL Sigma-Aldrich) imidacloprid (#37894), clothianidin (#33589), thiamethoxam (#37924), and thiacloprid (#37905). The doses tested were 1, 10 and 50 µg/L, which are all field relevant oral doses[19]. To ensure that the concentrations used were representative of the doses experienced in the field by pollinators, the lethality of 10 µg/L of imidacloprid was compared for bumblebees and *Drosophila.* In bumblebees, this concentration was shown to cause a mortality of 40% after 18 days[43]. In Drosophila, the same concentration of imidacloprid caused a mortality of 30% after 18 days (Appendix 1, Extended Fig.E1A). Thus the lethality of neonicotinoids appears to be similar between bumblebees and *Drosophila* when continuously self-dosing from a food source, suggesting *Drosophila* can be used to model the effects

of field relevant doses on behaviour. To compensate for the slightly greater resistance that was observed in Drosophila, a higher yet still field relevant concentration (50 μg/L) was tested in addition to 1 and 10 μg/L. For all experiments, both *Drosophila* and *B. terrestris*, insects continuously self-dosed from a food source of the stated concentration, as in the lethality assays and as would occur in the field. Neonicotinoid solutions and food were always kept wrapped in tinfoil to slow degradation.

In Chapter 3, three doses (1, 10 and 50 μg/L of imidacloprid, clothianidin, thiamethoxam and thiacloprid were tested, to fully assess the effects of these four common neonicotinoids on sleep and circadian rhythmicity. In Chapter 4, where the additive effects of neonicotinoid exposure were tested on various nAChr subunit *RNAi* mediated knockdown, 50 μg/L of imidacloprid and clothianidin were tested. A dose of 50 μg/L of either of these neonicotinoids was adequate to cause an effect on rhythmicity, activity or sleep in wildtype flies. As thiacloprid did not cause an effect on rhythmicity and thiamethoxam is a pro-drug for clothianidin[273], these were not tested. For the work carried out in Chapter 5 with *B. terrestris*, only imidacloprid was tested. This was because the adaptation of the DAM system for *B. terrestris*, the set-up of the RFID system, the quantity of raw data produced and the need to repeat experiments in multiple colonies to control for colony effects made carrying out experiments in *B. terrestris* much more time and labour intensive. Of the four neonicotinoids tested in *Drosophila*, imidacloprid was chosen for comparison in *B. terrestris* because it is the most commonly used neonicotinoid globally[23] and because the two species appear to have similar susceptibility to it when chronically exposed through their food source[43] (Appendix 1, Extended Fig.E1A).

Flies were maintained at 25°C, 55-65% humidity in a 12 hours light, 12 hours dark (LD) light cycle. For all experiments, virgin females were used. These were collected within 8 hours of eclosion and placed in vials containing fly food. Experimentation was carried out within 5 days of collection, which was performed using a CO_2 anaesthesia pad. Fly stocks were kept at 18°C. The fly stocks that were used and their sources are laid out in Table 2.1.

These stocks were used to create crosses, which are laid out in Table 2.2, through collection of virgins from one stock and males from the other. The use of the *UAS-GAL4* system allowed spatiotemporal targeted expression of transgenes.

Table 2.1: Fly stocks

Name	Description	Source/ Original Reference
iso^{31}	Wild type control	Gift from Prof. Ralf Stanewsky and Dr Maite Ogueta Gutierrez, University of Münster
	GAL4 Driver Lines	
tim-GAL4	Promoter line for expression in all clock neurons	Gift from Prof. Ralf Stanewsky, University of Münster
pdf-GAL4; Dcr-2; tubulin::GFP	Promoter line for expression in the PDF neurons, expresses GFP in the tubulin of these neurons, expresses dicer-2	Gift from Prof. Herman Wijnen, University of Southampton
	UAS Expression Lines	
UAS-nAChR Dα1 RNAi	*RNAi* mediated knock down of the Dα1 nAChR subunit	BDSC: #28688
UAS-nAChR Dβ2 RNAi	*RNA*i mediated knock down of the Dβ2 nAChR subunit	BDSC: #28038
UAS-nAChR Dα3 RNAi	*RNA*i mediated knock down of the Dα3 nAChR subunit	BDSC: #27671

Table 2.2: Genotypes used for experiments

Name	Description	Use
tim>Dα1 RNAi	*RNAi* mediated knock down of Dα1 nAChR subunit in the clock neurons	Circadian and sleep analysis
tim>Dβ2 RNAi	*RNAi* mediated knock down of Dβ2 nAChR subunit in the clock neurons	Circadian and sleep analysis
tim>Dα3 RNAi	*RNAi* mediated knock down of D*α3* nAChR subunit in the clock neurons	Circadian and sleep analysis
Pdf-GAL4; Dcr-2; tubulin::GFP> Dα1 RNAi	*RNAi* mediated knock down of and Dα1 GFP expression in the membrane of the PDF+ clock neurons	Quantification of s-LNv dorsal terminal axonal branching
Pdf-GAL4; Dcr-2; tubulin::GFP> Dβ2 *RNAi*	*RNAi* mediated knock down of and Dβ2 GFP expression in the membrane of the PDF+ clock neurons	Quantification of s-LNv dorsal terminal axonal branching
Pdf-GAL4; Dcr-2; tubulin::GFP> Dα3 RNAi	*RNAi* mediated knock down of and Dα3 GFP expression in the membrane of the PDF+ clock neurons	Quantification of s-LNv dorsal terminal axonal branching

For genotype behavioural experiments, *tim-GAL4* was used as a driver for the knockdown of neonicotinoid susceptible subunits. Within the brain, this driver drives expression within the clock neurons, the DN1s/2s/3s, the LNps, LNds and the LNvs (Fig.2.1A), allowing the role of different nAChR subunits within the central clock to be explored. For the immunohistochemistry experiments focusing on just the s-LNvs, the *PDF-GAL4* driver was used, which drives expression solely in the LNvs in the brain (Fig.2.1B). The nAChR subunits Dα1, Dα3 and Dβ2 were selected for investigation through *RNAi* mediated knockdown via *tim-GAL4*. Dα1 and Dβ2 have been shown to mediate neonicotinoid susceptibility in *Drosophila*[84] and Dα3 is rhythmically expressed in the LNvs and is a candidate for *rye,* which is an nAChR involved in sleep behaviour, suggesting it could play a role in the clock[86]. Thus, it was thought that these subunits may participate in the nAChRs involved in the effects of neonicotinoids on the clock and sleep. Expression maps for Dα1 and Dβ2 in the brain aren't yet available, making investigation into their potential occurrence and role in specific neuron groups, i.e. the clock neurons, novel.

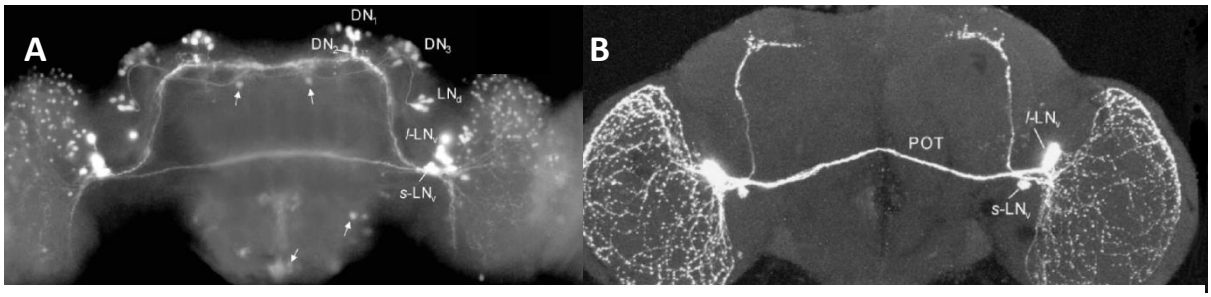

Figure 2.1: Expression patterns for *tim* and *PDF -GAL4* drivers in the *Drosophila* brain

A) The expression pattern for *tim-GAL4* in the brain, shown using *tim-GAL4;UAS-GFP* **B)** The expression pattern for *PDF-GAL4* in the brain, shown using *PDF-GAL4;UAS-GFP.* Images from Helfrich-Förster[147].

2.2 Bumblebee stocks

Bombus terrestris audax colonies (Biobest), containing cotton wool and approximately 80-100 workers, were ordered through Agralan (#BB121040-CF1). They were maintained at 21°C, 12:12 LD, with a 30 min dawn/dusk period where lights were at 50%. Colonies were provided with Biogluc® (Biobest) *ad lib* in the foraging arena and 1 teaspoon of pollen (Agralan #BB008513) every 5 days, into the nest box. Imidacloprid (PESTANAL Sigma-Aldrich #37894), was administered *via* the Biogluc®, at field relevant doses of either 1 or 10 µg/L[19]. Colonies were attached via a clear plastic tube (Ø15mm, 200 mm length), to a foraging arena (1000 × 50 × 50 mm) purpose built by the University of Bristol Mechanical Workshop out of UV transmitting acrylic. A wide ramp from the entrance to the foraging arena to the floor of the arena was built from card and duct tape to ensure that foragers could return to the nest box in darkness, when they cannot fly[248].

2.3 Climbing Assay in *Drosophila*

Climbing was measured using an adaptation[233] of the rapid iterative negative geotaxis (RING) method[249]. This utilises a fly's innate behaviour to move away from gravity when startled (negative geotaxis). Ten flies were placed into a vial of control or neonicotinoid food for 5 days. They were then flipped into an empty vial and given 5 min to acclimatise. Two sharp taps to the base of the vial knocked the flies to the bottom and then the number of flies who climbed above a line drawn at 7.5 cm within ten seconds was recorded (Fig. 2.2). This was repeated 25 times (with fresh flies). The locomotion assay was always carried out at the same time of day, ZT 2, as it has been shown that there may be circadian changes in negative geotaxis[250,251].

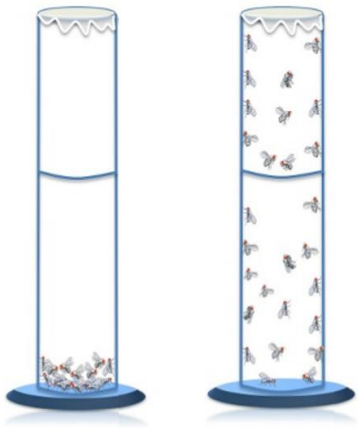

Figure 2.2: The climbing assay

Flies were tapped to the bottom of the vial and the number that managed to climb above 7.5cm in 10 seconds was recorded. Image from Madabattula et al[252].

2.4 Circadian Rhythm Assay in *Drosophila*

Circadian data was collected using the *Drosophila* Activity Monitor (DAM), (DAM2, Trikinetics Inc, USA)[120]. Individual flies were placed in DAM tubes (Trikinetics, PGT5x65) with control or neonicotinoid food and a rubber stopper (Trikinetics, CAP5-Black) in one end and cotton wool in the other. For each treatment group, 32 flies were tested. In the monitor an infrared beam intersected the tube and every beam break was counted in real time by a host computer (Fig.2.3). Each beam break was counted as a single activity bout, allowing the total activity for each fly to be summed per day and per 30 min bin for circadian analysis. The monitors were placed in an incubator and kept in LD conditions for 5 days, allowing entrainment, followed by 5 days of constant darkness (DD). Circadian data were collected for both the LD and the DD segments. The circadian data for the DD

segment provided information on the state of the endogenous clock and the data from the LD section showed how disruptions to the clock effected rhythmicity under more natural light conditions.

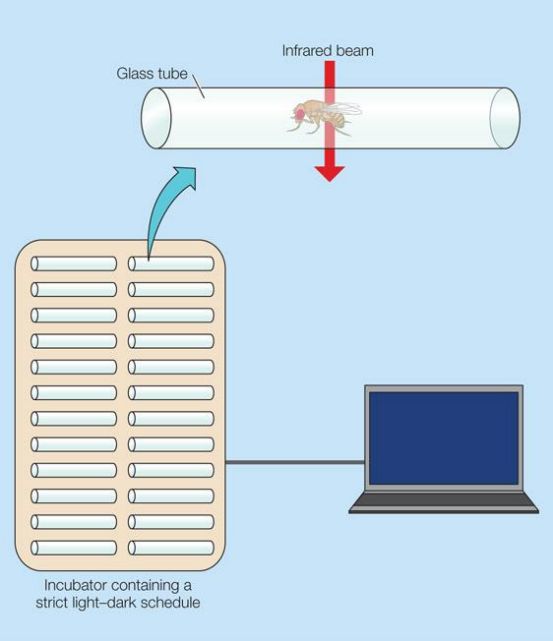

Figure 2.3- *Drosophila* Activity Monitor setup

The DAM setup used for circadian and sleep analysis in *Drosophila*. The fly is placed in an individual tube intersected with an infrared beam, put into a monitor with 31 other flies and the beam crosses for each fly are recorded by a host computer. Image from Garbe[253].

The host computer produced text files for each monitor containing the activity bouts for individual flies, summed into 30 min bins. These were cut to produce two files, one covering 9 am – 9 am for the 5 days of LD and another covering 9 am – 9 am for the 5 days of DD. After creating an actogram for each individual fly to provide a visualisation of rhythmicity (Fig. 2.4A), the LD and DD data were analysed separately. The data for any flies that did not survive until day 5 (*n* =0-13, depending on treatment group) were removed before analysis.

For each fly, the rhythmicity statistic (RS), period length and total daily activity levels were then calculated. Circadian analysis was carried out using *Flytoolbox*[254] in MATLAB (MATLAB and Statistics Toolbox Release 2015b, MathWorks, Natick, Massachusetts, United States). The rhythmicity statistic was calculated using conventional autocorrelation analysis. First, the data were filtered, using a low-pass Butterworth filter to remove any periodicities of less than 4 hours. The dataset was then paired with itself, gradually moved out of register with itself and the correlation coefficient plotted. At 0

hours, the two data sets are identical, and then, for rhythmic data, they return to high correlation approximately every 24 hours. The value of the third peak in the auto correlogram is reported as the rhythmicity index (RI), a statistical representation of the rhythmicity of the fly's activity. The RS is calculated as the ratio of this RI to the value of the 95% confidence line (Fig. 2.4B). Conventionally, an RS above 2 is rhythmic, an RS between 1.5 and 2 is weakly rhythmic and an RS equal to or less than 1.5 is arrhythmic[254]. The period length is also calculated from the auto correlogram, by calculating the time between the peaks in correlation (Fig. 2.4B).

The day and night activity levels for each fly were calculated using the *daynight* program[255] in MATLAB. The individual fly's period length was used to split the activity data into subjective days and nights and then the activity counts for each were summed. The mean daily activity in daytime and night-time for the 5 days was then calculated.

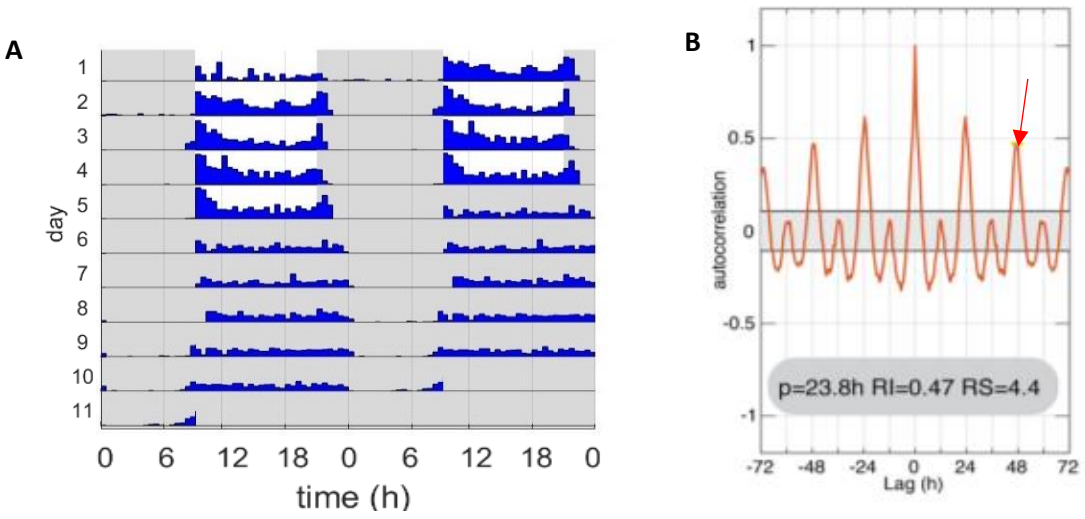

Figure 2.4: Circadian rhythmicity analysis

A) The activity of a single fly in the DAM for ten days. Each blue bar represents the number of activity counts (beam crosses) that occurred within that 30 min bin. A white background indicates lights on whilst a grey background indicates lights off. Activity is double plotted, meaning each day is plotted once on the right half of the graph and then repeated again on the row directly below, on the left half. This allows easier comparison of each day with the one preceding and following it and makes rhythms in activity easier to identify. Lights were on 9am-9pm for the first 5 days, followed by 5 days constant darkness. In this example actogram, most of the activity clearly occurs within the day in LD and continues to occur within the subjective day in DD.

B) An example auto correlogram with an arrow indicating the third peak, from which the RI and RS are calculated.

The rhythmicity statistic is affected by environmental variables, for example vibrations from ongoing building works in the building in which experiments were conducted. This led to differences in the mean RS in control flies between different experiments. For this reason, treatment flies were only compared to control flies placed into the DAM concurrently. In Chapter 3, imidacloprid and thiacloprid were run together with a control and clothianidin and thiamethoxam were run together with a different control. All of the genotypes in Chapter 4 were tested simultaneously, alongside the neonicotinoid exposed Dα3 knockdowns. The neonicotinoid exposed Dα1 and Dβ2 knockdowns were tested separately with separate controls.

2.5 Analysis of *Drosophila* Sleep Behaviour

Sleep data was also collected using the DAM setup, as above. In flies, sleep is defined as any inactivity lasting more than 5 min[168]. Sleep analysis was carried out on the 5 days of LD[256], using activity data that had been summed into both 1 min and 30 min bins. Analysis was performed using the Sleep and Circadian Analysis MATLAB Program[257] (SCAMP). The mean total quantity of sleep per 30 min bin was calculated and displayed as demonstrated in Fig. 2.5. Total quantity of sleep for the day and night for each fly was quantified and the mean taken for the 5 days. Other sleep measures reported were the number of sleep episodes initiated during the day and night and the mean length of these episodes. Latency was also calculated, which is the time taken after a change in light conditions (lights on or lights off) before the first sleep episode was initiated. Again, for all of these measures, the daily mean was taken for the 5 days of data collected.

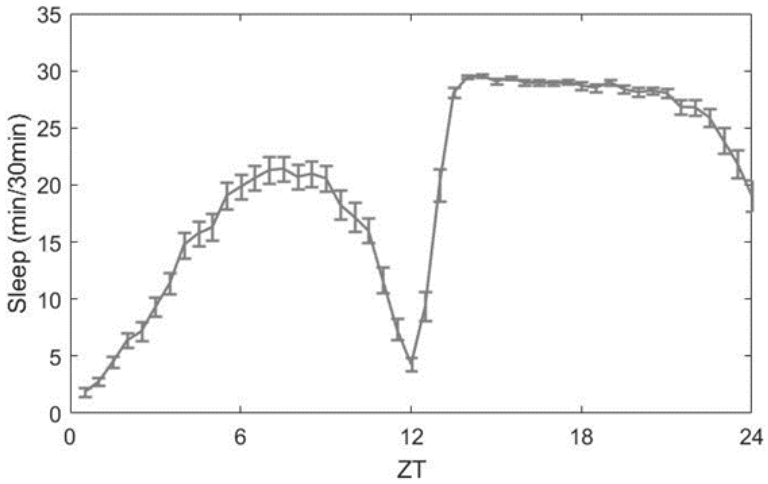

Figure 2.5: Sleep graph

The mean daily sleep of the 32 flies held in one DAM monitor, averaged for five days of LD. Each point shows the sleep (min) achieved during that 30 min bin (Mean ± Standard Error of the Mean (SEM). The x-axis shows *zeitgeber* time (ZT), indicating the number of hours since the *zeitgeber* (lights on). Thus ZT 0-12 represents lights on and ZT 12-24 represents lights off. In this sleep graph for a control fly, the long night-time sleep starting just after lights off and the siesta that occurs in the middle of the day are both clearly visible.

2.6 Circadian rhythms and sleep assays for bumblebees

For the collection of circadian and sleep data for bumblebees, a system very similar to the DAM was setup, using the larger Locomotor Activity Monitor (LAM), (LAM16, Trikinetics Inc, USA)[208]. Foragers were collected from the foraging arena and loaded into tubes (PGT16x100). At one end of the tube was a rubber cap (CAP16-Black) with a silica packet (celloexpres, #SG_1g) glued inside to control the humidity. At the other end, a 15mL falcon tube with a small whole drilled near the base was attached. This contained Biogluc® with or without neonicotinoids, allowing bees to feed *ad lib* throughout the experiment (Fig. 2.6). Data was collected and analysed in the same way as for the DAM data.

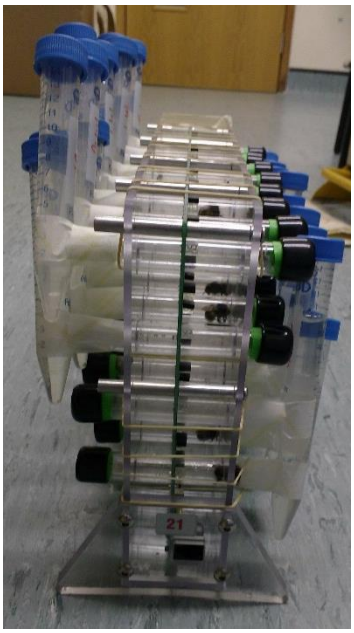

Figure 2.6: The Locomotor Activity Monitor setup for bumblebees

Image of the LAM setup for circadian and sleep analysis in bees. Foragers are in glass tubes with a stopper at one end and a falcon tube containing Biogluc® at the other.

2.7 Foraging rhythmicity assay for bumblebees in the colony

Circadian rhythmicity within the colony was assayed using a micro radio frequency identification (RFID) setup[258]. Approximately 40 foragers were collected from the foraging arena. These were anaesthetised using CO_2 and an RFID tag (Microsensys GmbH mic3-TAG) was stuck to the centre of their thorax with superglue (Loctite) as in Fig. 2.7. Foragers were then returned to the colony nest box. After a day for acclimatisation and recovery from the CO^2 exposure, recording began. An RFID reader (Microsensys GmbH iID®MAJAreadermodule4.1) was placed at the entrance to the foraging arena so that foragers had to pass through the reader to enter the arena. The data from the readers were collected on a host (Microsensys GmbH iID®HOSTtypeMAJA4.1), for a 10 day period; 5 days LD

and 5 days DD, as in the DAM experiments above. The data were then summed into 30 minutes (min) bins, with each pass through the reader being counted as a single activity bout. These data were then analysed as above using the *Flytoolbox* in Matlab[259]. This was repeated with 3 colonies for each treatment group. Pollen was provided the day before recording began and on day 5. Biogluc was available *ad lib* in the foraging arena and was either untreated for control groups or dosed with 10 µg/L imidacloprid for treated groups.

Figure 2.7: Radio Frequency Identification in foragers

Image showing bumblebee foragers with unique RFID tags attached to their thoraxes.

2.8 Immunohistochemistry

2.8.1 Sample preparation

Quantification of the arborisation and PDF accumulation at the s-LNv dorsal terminals was performed by immunohistochemistry and confocal imaging[147]. Flies were placed in vials containing either standard food or standard food containing neonicotinoids and kept in LD conditions for 5 days. Flies were then anesthetised at either ZT 2 (2 hours after lights on) or ZT 14 (2 hours after lights off) and decapitated. Heads were fixed in phosphate-buffered solution (PBS) with 4% paraformaldehyde (PFA), (Thermo Fisher Scientific) containing 0.008% Triton X-100 (Sigma-Aldrich) for 45 min at room temperature. Heads were washed quickly twice in PBS with 0.5% Triton X-100 (PBT 0.5%), followed by three 20 min washes in PBT 0.5% and then the brains were dissected out in PBT 0.1%. Brains were then blocked in 5% Normal Goat Serum (NGS), (Thermo Fisher) for 1 hour. Brains were placed in NGS containing the primary antibodies (Table 2.3) and incubated at 4°C for 36 hours.

Brains were then washed again as above. They were then placed in NGS containing the secondary antibodies (Table 2.3) and left to incubate at room temperature for 3 hours, followed by 24 hours at

4°C. Brains were washed again. They were then mounted onto glass slides using spacers (SecureSeal™, Grace Bio-Labs #654002) and covered with VectaShield hard set medium (Vector Laboratories). After 30 min, cover slides were secured with CoverGrip (Biotium #23005) and slides were stored at 4°C. At every stage samples were kept covered with aluminium foil to keep them from the light.

Table 2.3: Antibodies for immunohistochemistry

Primary Antibodies	Concentration
Mouse monoclonal anti-PDF (Developmental Studies Hybridoma Bank, #PDF-C7)	1:200
Rabbit polyclonal anti-GFP (Life Technologies # A11122)	1:1000
Secondary Antibodies	
Alexa Fluor Plus 555 Goat anti-mouse (Life Technologies # A32723)	1:1000
Alexa Fluor Plus 488 Goat anti-rabbit (Life Technologies # A32732)	1:100

2.8.2 Imaging and Analysis

Imaging was carried out on a Leica SPE confocal laser scanning microscope with the green channel imaged at 480 – 551 nm and the red at 571 – 650 nm. Z stacks were captured of the s-LNv dorsal terminals using a 64× oil immersion objective, with a step size of 2 µm. For the red channel, which was capturing PDF staining intensity, the laser was set at 30%, gain (V) at 775 and offset at 0. These conditions were kept the same for all images collected. Maximal projection stacks were created for both GFP and PDF staining and analysed using the image processing package *FIJI* in ImageJ[260].

The arborisation of each s-LNV terminal was calculated from the GFP images using an adaptation[147] of the Scholl analysis[261]. A centre point was positioned at the first branching point of the terminal with 6 concentric circles emanating from it, 10 µm apart (Fig. 2.8). Every branch that crossed one of these circles was marked as one cross and the total number of crosses for each terminal was calculated. For each brain both the right and the left s-LNv dorsal terminals were analysed.

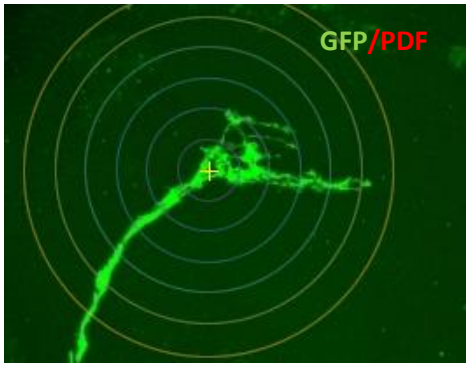

Figure 2.8- Scholl analysis

Example image showing how Scholl analysis of the dendritic arborisation of the s-LNv dorsal terminals was carried out. Concentric circles were placed 10 µm apart, starting from the first branching point. The number of points at which an axonal branch crossed each line was summed, quantifying the degree of axonal branching.

To quantify PDF accumulation, the maximal projection image of the PDF staining was analysed using *FIJI* in ImageJ[260]. The image was cut at the first branching point to create an image containing only the terminals and not the cell axon, as in Fig. 2.9A-B. The threshold was then adjusted to create a black and white image. The despeckle filter was used to reduce noise and watershed segmentation carried out to separate the different PDF compartments (Fig 2.9C). This image was used as a template for calculating the PDF staining in the original maximal projection image, allowing the PDF staining intensity to be calculated for each of these compartments and the mean taken. The mean of both hemispheres of the brain was calculated and reported.

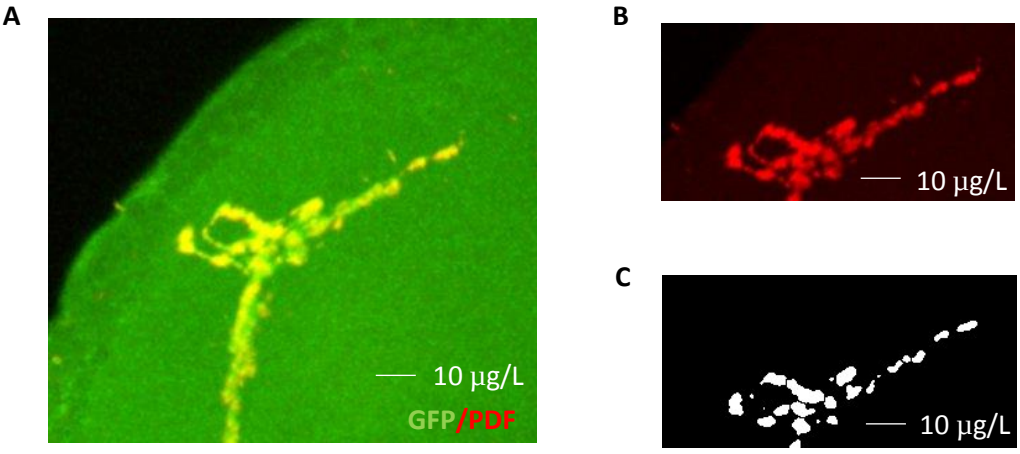

Figure 2.9- PDF accumulation analysis

Example images showing how PDF accumulation was calculated. **A)** Image of the neuron, showing GFP in green and PDF in red, overlaid, **B)** Image showing the PDF staining of the terminals, **C)** Image showing the template for calculating the intensity of each PDF compartment in the terminals

2.9 Statistical analysis

For the climbing, circadian and sleep data, analysis was carried out using a one-way ANOVA. First the data was checked for normality using a Shapiro-Wilk test. The homogeneity of variance was also tested using Levene's test for equality of variances. For data that met both of these assumptions, means were then compared using a one-way ANOVA with *post hoc* pairwise comparisons using Tukey's multiple comparisons test.

The sleep data failed both of these assumptions. Thus, permutation tests[262] were conducted in R 3.4.1[263] . As the resulting *p* values closely matched those produced by analysing the same data using a one-way ANOVA as above, and because ANOVA is relatively robust to deviations from normality when sample sizes are large[264] the results of the one-way ANOVA were displayed.

The immunohistochemistry data and comparison LD to DD controls for isolated foragers were analysed using Pearson's T-test.

Statistical analysis was done in IBM SPSS Statistics 24 (IBM Corporation, USA). Graphs were created in GraphPad (Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA). For all histograms, every data point was plotted with lines showing the mean ± Standard Error of the Mean (SEM). Where *post hoc* tests were done, these were displayed as $p \leq 0.05$ *, $p \leq 0.01$ **, $p \leq 0.001$ ***, $p \leq 0.0001$ ****).

3

Chapter 3: The behavioural effects of neonicotinoid pesticides on locomotion, rhythmicity and sleep in *Drosophila*

As illustrated in Chapter 1, *Drosophila* provide an excellent model organism in which to investigate circadian rhythmicity and sleep. In this chapter, *Drosophila* are utilised to thoroughly explore the effects of neonicotinoid pesticides on the behavioural rhythmicity and sleep of insects. Section 3.2-3.4 will cover the effects of neonicotinoids on the mobility, activity, rhythmicity and period length of flies, whilst section 3.5 will detail the effects on sleep quantity and quality. The results laid out in 3.2-3.5 will then be discussed in section 3.6, with the conclusions summarised in section 3.7.

3.1 Introduction

Drosophila have been utilised for circadian and sleep research for decades, leading to the identification of key clock genes[97] and elucidation of the clocks molecular[124] and membrane components[162]. Thanks to this extensive research, we know that ACh signalling via nAChRs is a vital component of the clock and sleep/wake circuitry. The LNvs, the key pacemaker cells of the clock and the arousal cells of the sleep/wake circuit, rely upon nAChR signalling to maintain synchronicity and rhythmic activity[92] and to receive information from light sensing organs[90,91]. Thus, circadian rhythmicity and sleep present potential off target behavioural effects for neonicotinoids that have not been explored. In this chapter the effects of imidacloprid, clothianidin, thiamethoxam and thiacloprid on numerous measures of rhythmicity and sleep are characterised and discussed.

3.2 Neonicotinoids reduced climbing ability

Climbing ability was assayed using the negative geotaxis methodology as laid out in Chapter 2. All three of the banned neonicotinoids tested caused a reduction in locomotion (Fig. 3.1). For imidacloprid (Fig. 3.1A), clothianidin (Fig. 3.1B) and thiamethoxam (Fig. 3.1C) this effect was observed at both 10 and 50 µg/L whilst 1 µg/L of any neonicotinoid had no effect. For thiacloprid (Fig. 3.1D) none of the doses tested had an effect.

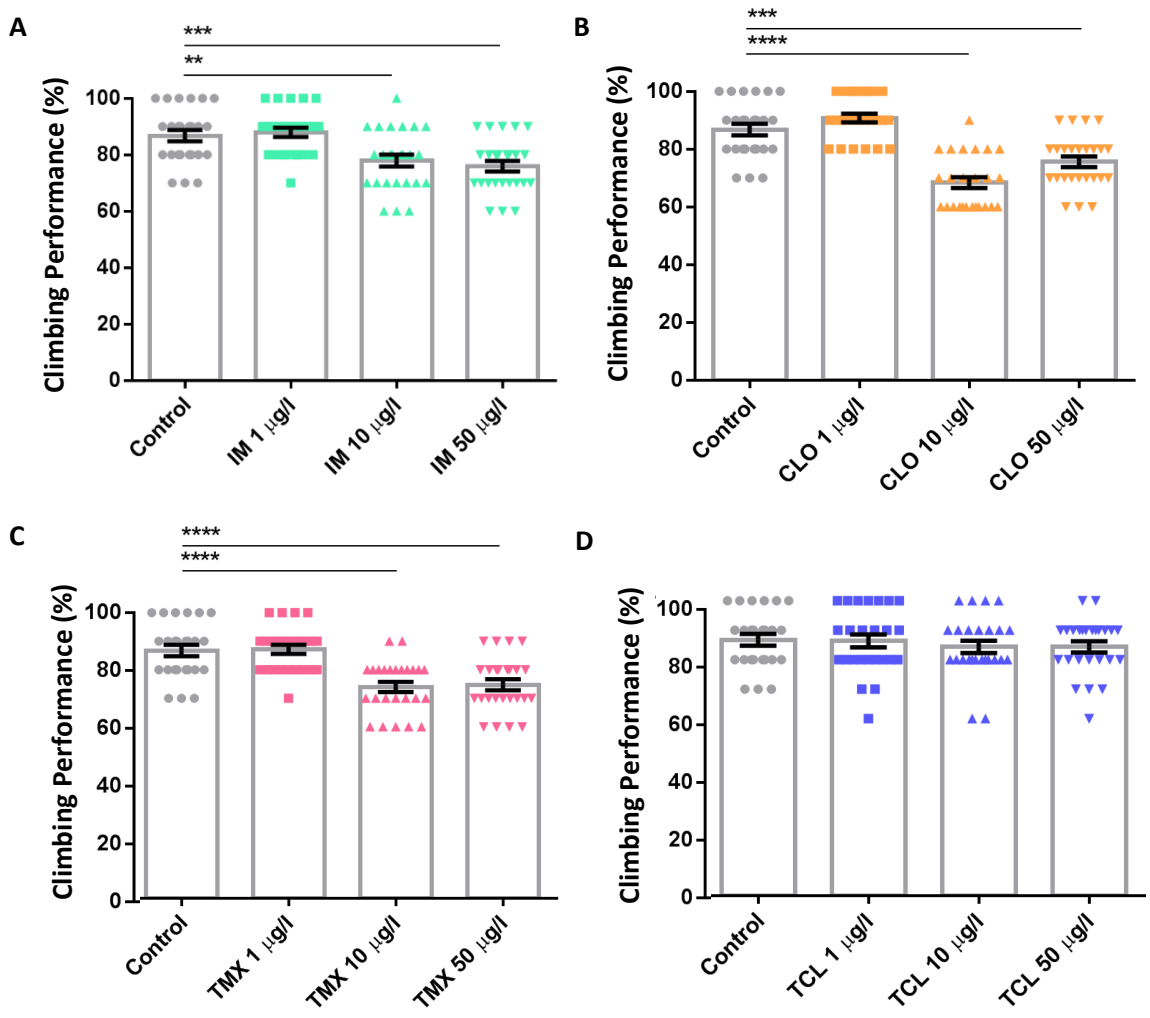

Figure 3.1: Field relevant doses of neonicotinoids reduce locomotor performance

Climbing performance (%) for flies exposed to 1, 10 or 50 µg/L of **A)** imidacloprid (IM) ($F_{(3,96)}$=9.9, p≤0.001), **B)** clothianidin (CLO) ($F_{(3,96)}$=32.0, p p≤0.0001), **C)** thiamethoxam (TMX) ($F_{(3,96)}$=15.8, p p≤0.001) and **D)** thiacloprid (TCL) ($F_{(3,96)}$=0.388, p=0.762). Each data point represents ten flies tested together; 25 repeats were carried out for each treatment.

3.3 Neonicotinoids affected behavioural rhythmicity in constant darkness

3.3.1 Neonicotinoids tended to increase total activity in constant darkness

To further assess the effects of neonicotinoids on locomotion, the total activity levels of individual flies during the DAM assay were recorded. Neonicotinoid exposure was more likely to cause an increase in activity than a decrease, as can be seen in Fig. 3.2. Thiamethoxam (Fig. 3.2C) exposure caused an increase in activity in the subjective daytime at 1 µg/L and clothianidin (Fig. 3.2B) exposure caused an increase in activity in the subjective night-time at both 10 and 50 µg/L. Only imidacloprid (Fig. 3.2A) exposure caused a decrease, with 50 µg/L reducing activity in the subjective daytime. Thiacloprid (Fig. 3.2D) exposure had no effect on activity levels at the doses tested.

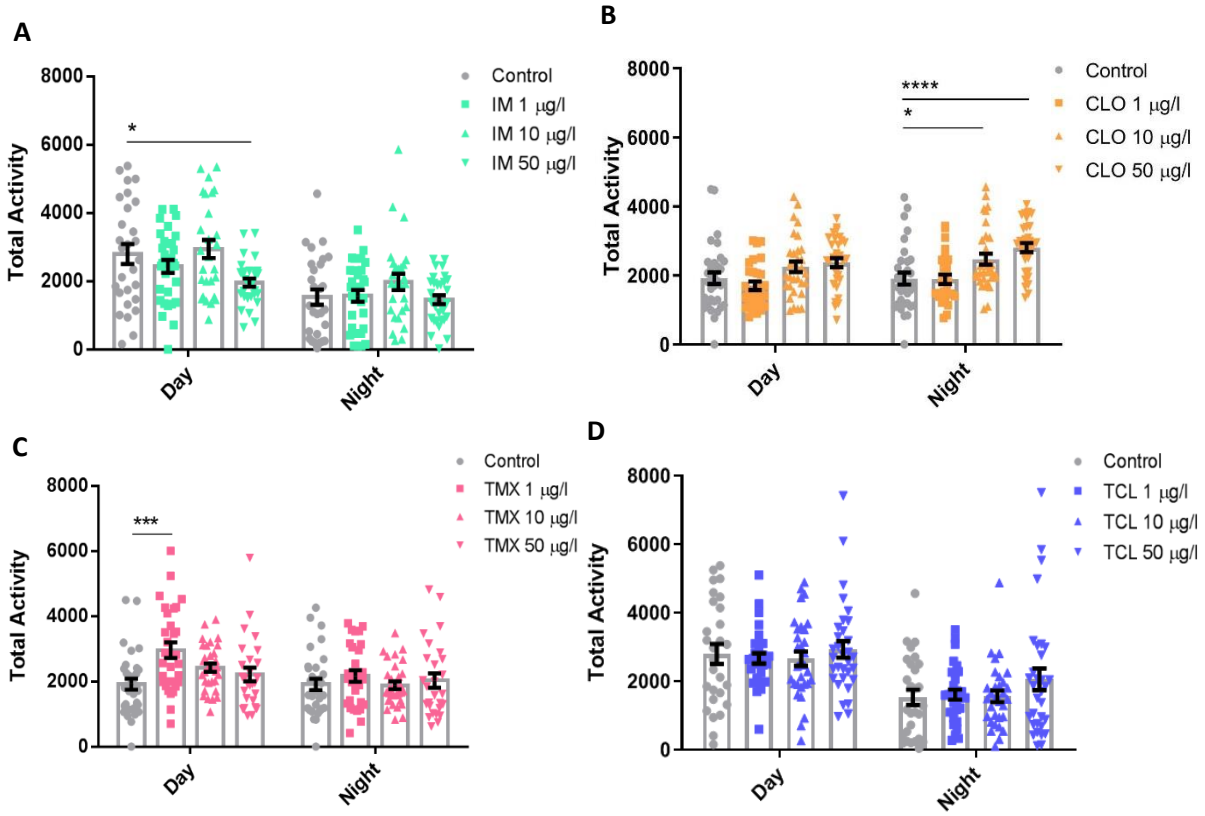

Figure 3.2: Neonicotinoids effect activity levels in continuous darkness

Total activity counts for the day and night for flies exposed to 1, 10 or 50 µg/L of **A**) imidacloprid (IM), in day ($F_{3,112}$=3.9, p=0.011) and night ($F_{3,112}$=1.4, p=0.255), **B**) clothianidin (CLO), day ($F_{3,120}$=4.1, p=0.008) and night ($F_{3,120}$=8.8, p≤0.001), **C**) thiamethoxam (TMX) day ($F_{3,116}$=5.5, p=0.001) and night ($F_{3,116}$=0.6, p=0.629) or **D**) thiacloprid (TCL), day ($F_{3,118}$=1.2, p=0.299) and night ($F_{3,118}$=0.8, p=0.514). Each data point represents a single fly, n=28-32 flies per treatment.

3.3.2 Neonicotinoids reduced the strength of behavioural rhythmicity in constant darkness

The behavioural rhythmicity of flies was then tested during chronic exposure to neonicotinoids. As can be seen from the representative actograms in Fig. 3.3, the three banned neonicotinoids caused a dose dependant breakdown in behavioural rhythmicity whilst thiacloprid did not. The flies all appear to be able to entrain, maintaining visible rhythmicity in LD conditions, with most activity occurring during the 12 hours of light. However, once placed into constant darkness, for many of the doses tested this rhythm broke down. Activity can then be seen occurring throughout both the subjective day and night, unlike in control flies who are able to maintain behavioural rhythmicity after the removal of environmental cues.

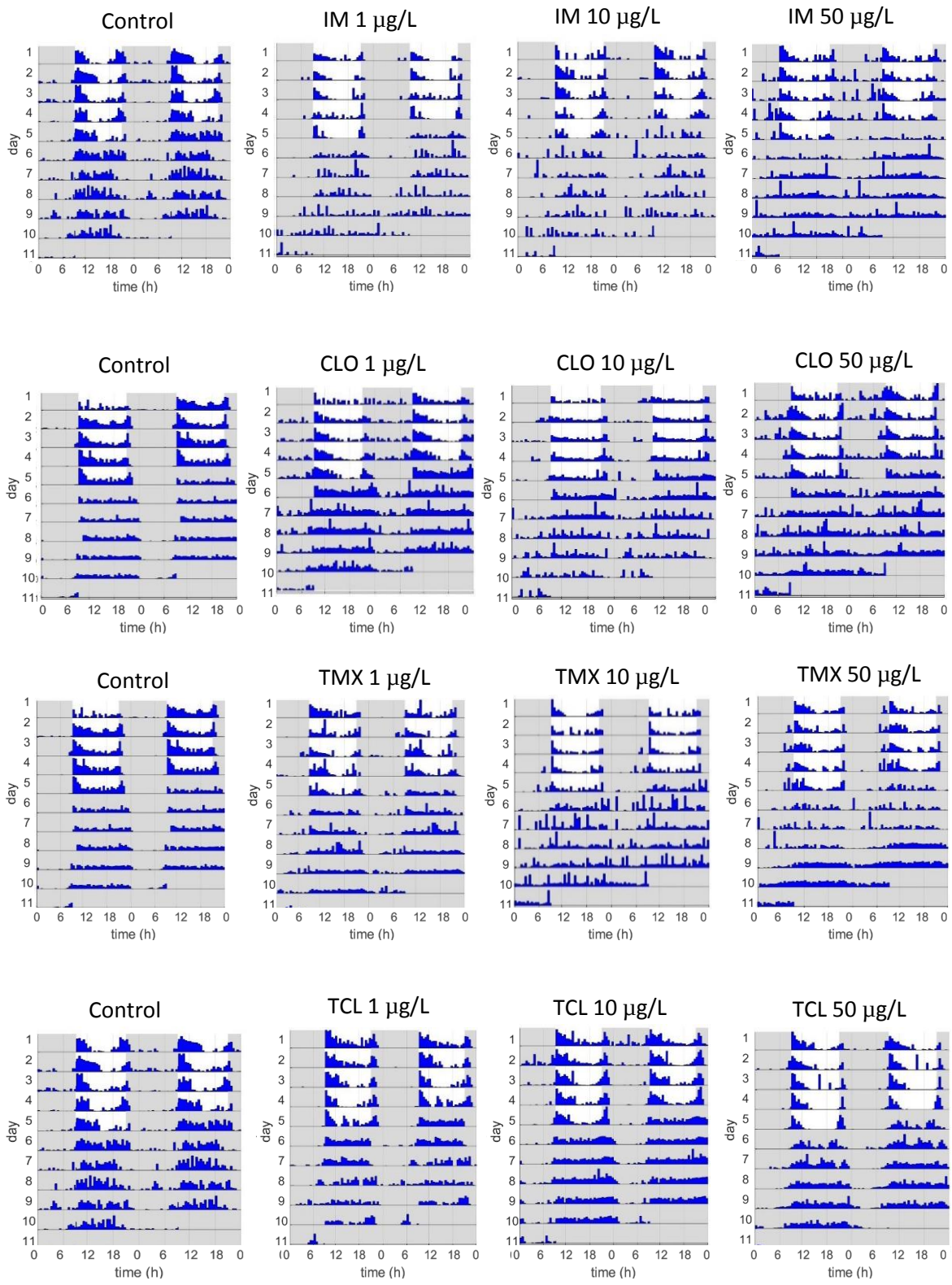

Figure 3.3: Neonicotinoids disrupt behavioural rhythmicity

Representative actograms for control flies, compared with flies exposed to 1 µg/L, 10 µg/L or 50 µg/L of imidacloprid, clothianidin, thiamethoxam or thiacloprid.

3.3.3 Neonicotinoids reduced the rhythmicity statistic in constant darkness

As seen above, neonicotinoid exposure caused a breakdown of rhythmicity once light signals were removed. This effect was quantified by calculating the rhythmicity statistic (RS) for the flies in each treatment group for the five days of constant darkness. The rhythmicity statistic is one of the key measures of behavioural rhythmicity, as explained in Chapter 2. Conventionally, an RS above 2 indicates strong rhythmicity, an RS between 1.5 and 2 shows weak rhythmicity and an RS of 1.5 or below indicates arrhythmicity[259].

The three banned neonicotinoids caused a dose dependant reduction in rhythmicity strength, as can be seen in Fig. 3.4. Thiamethoxam (Fig. 3.4C) caused the most dramatic effect, with a reduction in rhythmicity occurring for every dose tested (1-50 µg/L). Clothianidin (Fig. 3.4B) exposed flies showed a reduction in rhythmicity for 50 µg/L. For imidacloprid (Fig. 3.4A), although the one-way ANOVA was borderline-significant, *post hoc* tests showed that flies on 50 µg/L had significantly lower mean rhythmicity than control flies. For these three neonicotinoids, exposure to 50µg/L had a similar effect, bringing the mean rhythmicity statistic to near 1.5. Again, thiacloprid (Fig. 3.4D) showed no effect.

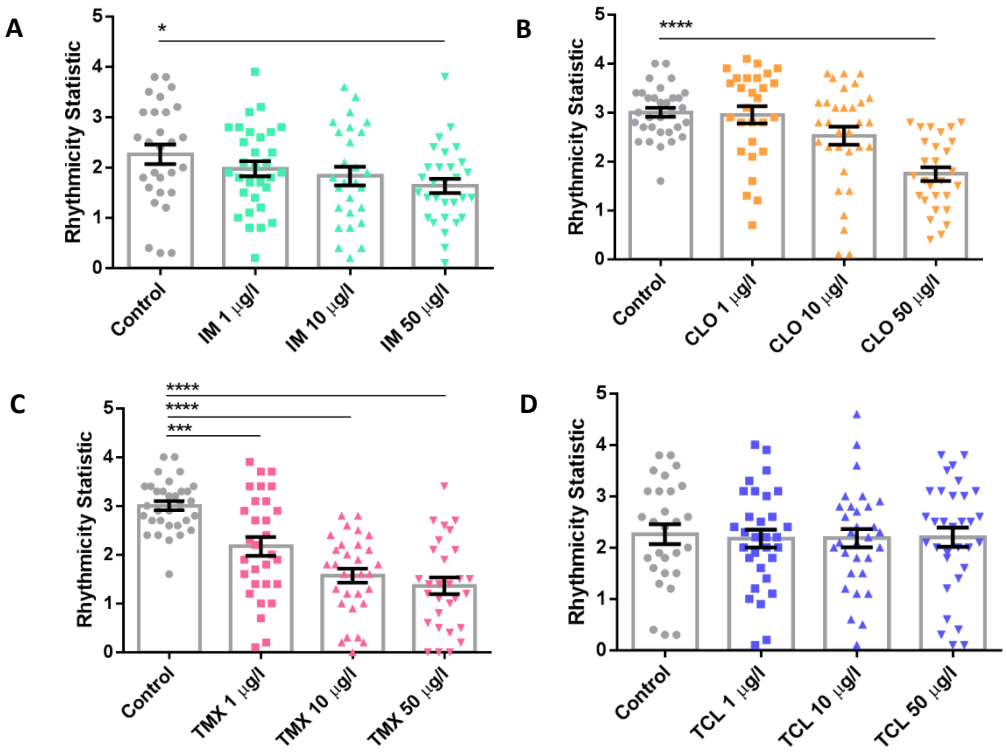

Figure 3.4: Neonicotinoids reduced behavioural rhythmicity in continuous darkness

Mean behavioural rhythmicity strength (rhythmicity statistic) for flies exposed to 1, 10 or 50 µg/L of **A)** imidacloprid (IM) ($F_{3,112}$=2.5, p=0.061), **B)** clothianidin (CLO) ($F_{3,116}$=14.2, p≤0.001), **C)** thiamethoxam (TMX) ($F_{3,118}$=23.7, p≤0.001) and **D)** thiacloprid (TCL) ($F_{3,118}$=0.05, p=0.987). Each data point represents a single fly, n=28-32 flies per treatment.

3.3.4 Neonicotinoid exposure increased the proportion of flies that were arrhythmic in constant darkness

Arrhythmia represents the most extreme disruption of rhythmicity, so the proportion of the population experiencing total loss of rhythmicity (RS≤1.5) was quantified (Fig. 3.5). As with the mean rhythmicity data, the effect for the three banned neonicotinoids was dose dependant and thiamethoxam showed the greatest effect size, with 65% of flies arrhythmic at 50 µg/L. This was followed by clothianidin, which caused arrhythmicity in 36% of flies at 50 µg/L, and imidacloprid with 27% arrhythmicity at 50 µg/L.

A dose of 1 µg/L of imidacloprid or clothianidin caused 8% and 11% of flies respectively to become arrhythmic and 10 µg/L of either caused an increase of 19%. A dose of 1 µg/L or 10 µg/L of thiamethoxam caused increases in arrhythmicity of 10% and 23% respectively. Thiacloprid exposure caused a very small increase in arrhythmicity, between 1 and 4%, which was not dose dependant. Of these, only 1 µg/L of imidacloprid or 1-50 µg/L of thiacloprid did not cause a significant increase.

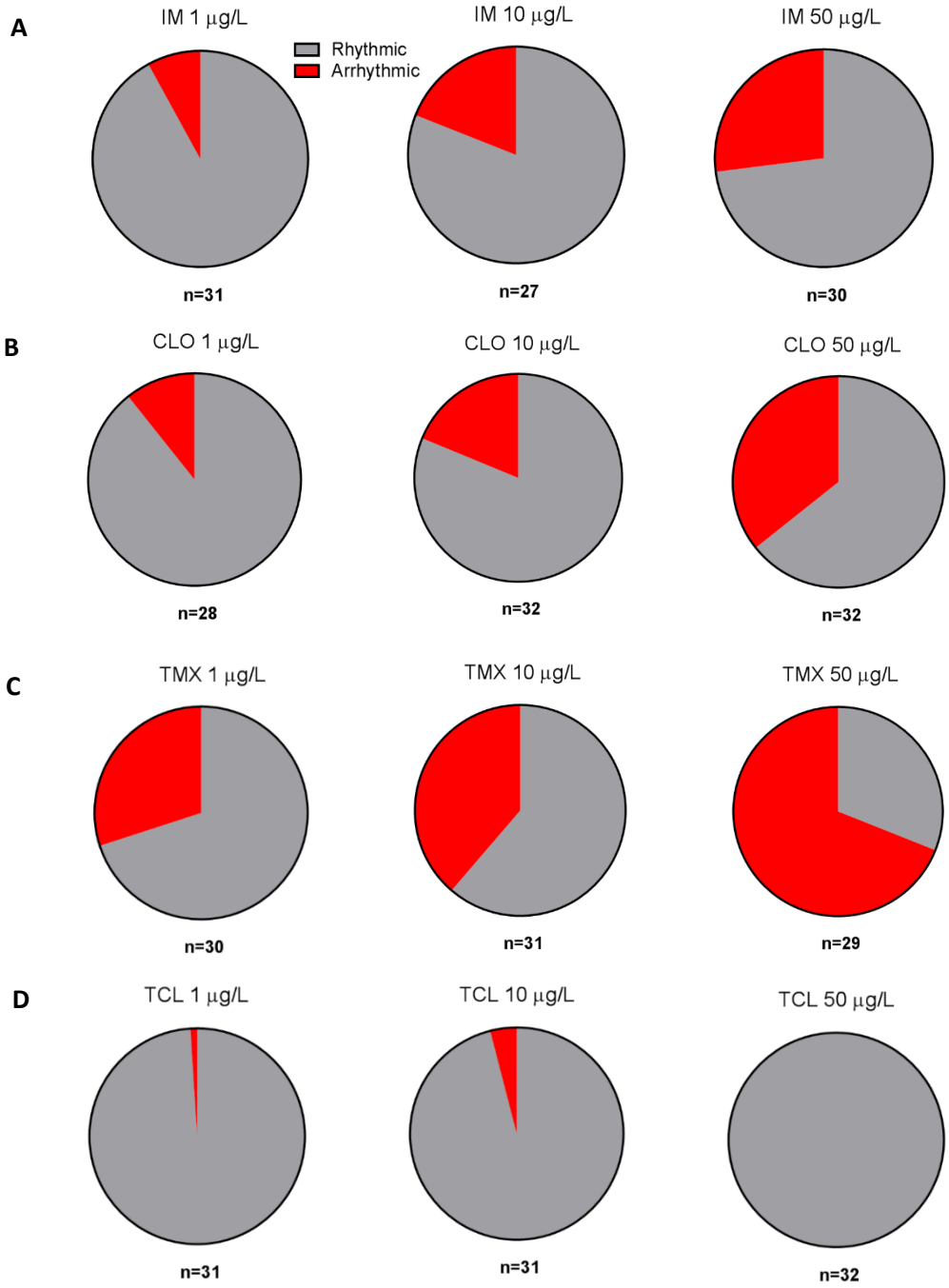

Figure 3.5: Neonicotinoids increase the proportion of flies that were arrhythmic in constant darkness

Pie charts showing the increase in the proportion of the population who were arrhythmic (RS≤1.5) compared to controls for **A)** 1 µg/L (χ^2_1=1.9, p=0.160), 10 µg/L (χ^2_1=10.2, p=0.001) and 50 µg/L (χ^2_1=18.4, p≤0.001) of imidacloprid (IM), **B)** 1 µg/L (χ^2_1=10.6, p=0.001), 10 µg/L (χ^2_1=20.0, p≤0.001) and 50 µg/L (χ^2_1=42.9, ≤0.001) clothianidin (CLO), **C)** 1 µg/L (χ^2_1=35.9, p≤0.001), 10 µg/L (χ^2_1=47.4, p≤0.001) and 50 µg/L (χ^2_1=104.3, p≤0.001) thiamethoxam (TMX) and **D)** 1 µg/L (χ^2_1=0.8, p=0.400), 10 µg/L (χ^2_1=1.9, p=0.160) and 50 µg/L (χ^2_1=0.470, p=0.5) thiacloprid (TCL). n=27-32 flies per treatment.

3.3.5 Neonicotinoids had no effect on period length in constant darkness

Another common measure of changes to rhythmicity is the period length. Often changes in rhythmicity can result in changes in period length, with flies developing shorter or longer days or ultradian patterns. However, none of the neonicotinoids tested showed any change in period length compared to control flies, all remaining around 24 hours in length (Fig. 3.6).

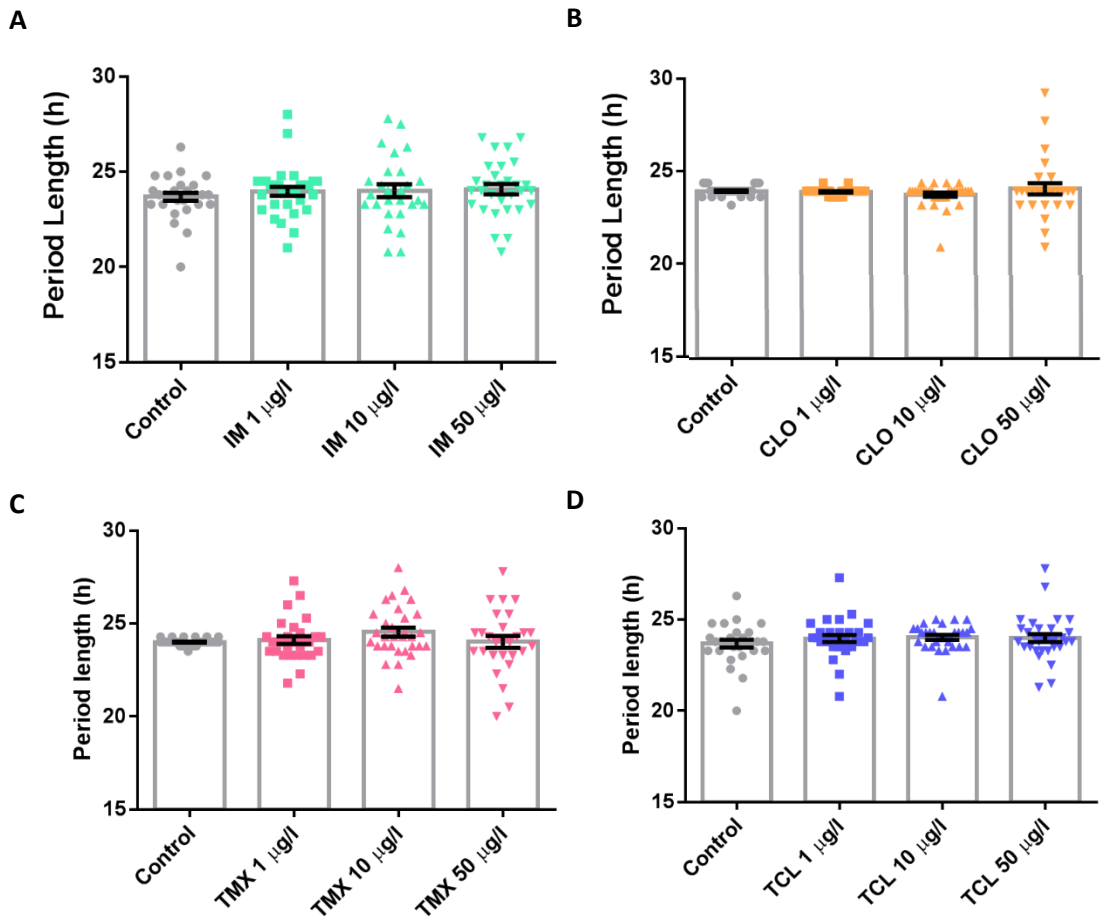

Figure 3.6: Neonicotinoids do not affect period length in constant darkness

Mean period length for flies exposed to 1, 10 or 50 µg/L of **A)** imidacloprid (IM) ($F_{3,112}$=0.4, p=0.722), **B)** clothianidin (CLO) ($F_{3,116}$=0.7, p=0.536), **C)** thiamethoxam (TMX) ($F_{3,116}$=1.5, p=0.207) and **D)** thiacloprid (TCL) ($F_{3,118}$=0.7, p=0.584).

Each data point represents a single fly, n=28-32 flies per treatment.

3.4 Neonicotinoids affected behavioural rhythmicity under light:dark conditions

In order to assess whether the breakdown of rhythmicity observed in constant darkness might affect behavioural rhythmicity in the field, rhythm strength was also assessed under field relevant light conditions, with 12 hours of light and 12 hours of dark (LD) per day.

3.4.1 Neonicotinoids weakened behavioural rhythmicity under light:dark conditions

Fewer of the neonicotinoids tested reduced mean rhythmicity in LD conditions (Fig 3.7). Imidacloprid (Fig. 3.7A) and thiamethoxam (Fig. 3.7C) both caused a reduction in rhythm strength, with this reduction visible at 10 and 50 µg/L in thiamethoxam exposed flies and 50 µg/L in imidacloprid exposed flies. Despite these reductions, mean rhythmicity statistic remained well above 1.5 for all groups suggesting that the majority of flies were still strongly rhythmic. Neither clothianidin (Fig. 3.7B) nor thiacloprid (Fig. 3.7D) affected mean rhythmicity strength at any of the doses tested.

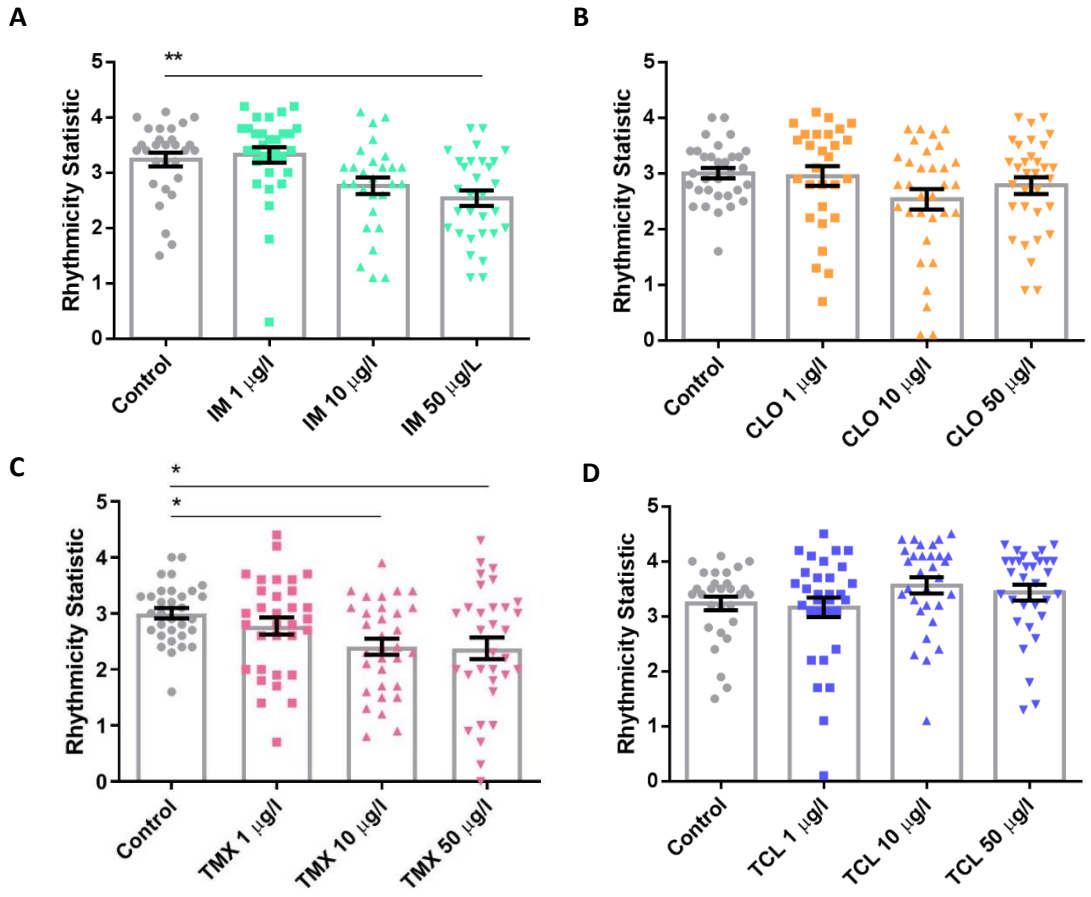

Figure 3.7: Neonicotinoids affect behavioural rhythmicity in light dark conditions

Mean behavioural rhythmicity strength (rhythmicity statistic-RS) for flies exposed to 1, 10 or 50 µg/L of **A)** imidacloprid (IM) ($F_{3,114}$=7.3, p≤0.001), **B)** clothianidin (CLO) ($F_{3,120}$=1.9, p=0.127, **C)** thiamethoxam (TMX) ($F_{3,124}$=4.0, p=0.009) and **D)** thiacloprid (TCL) ($F_{3,119}$=1.5, p=0.217). Each data point represents a single fly, n=28-32 flies per treatment.

3.4.2 Neonicotinoids increased the proportion of flies that are arrhythmic under light:dark conditions

Although many of the neonicotinoid doses tested had no effect on mean rhythmicity, every dose significantly increased the proportion of the population that became arrhythmic, except 1 µg/L of imidacloprid or 10-50 µg/L of thiacloprid (Fig 3.8). This effect was greatest for 10 µg/L of clothianidin and 10 and 50 µg/L of thiamethoxam, all of which caused an increase in arrhythmicity of 19% compared to control flies. A dose of 1 µg/L of thiamethoxam or clothianidin, or of 10 to 50 µg/L of imidacloprid, caused an increase in arrhythmicity of approximately 10%. Exposure to 1 µg/L imidacloprid or to thiacloprid had the least effect, causing an increase of between 3-7% depending on dose.

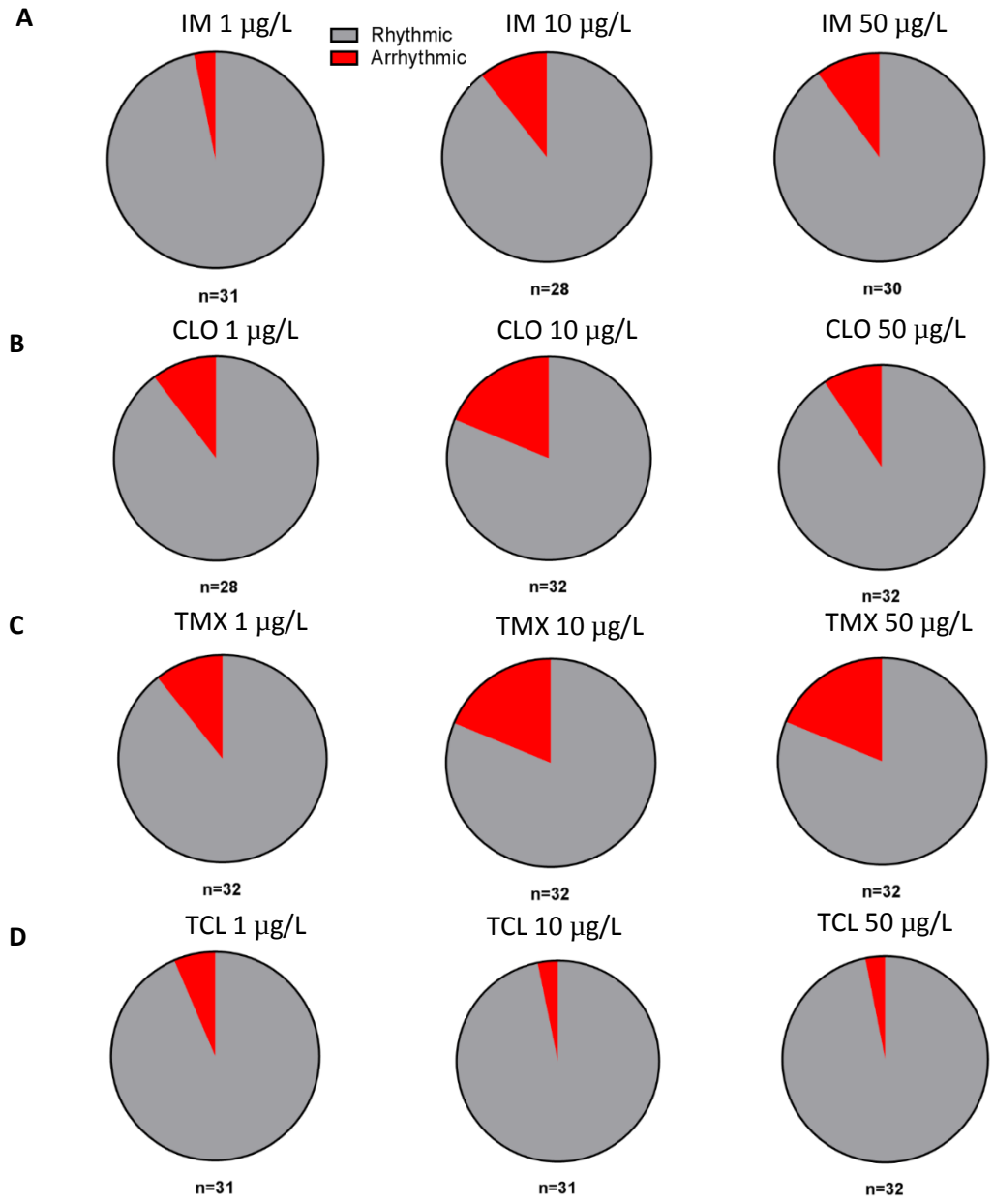

Figure 3.8: Neonicotinoids increase the proportion of flies that were arrhythmic under light-dark conditions

Pie charts showing the increase in the proportion of the population who were arrhythmic (RS≤1.5) compared to controls for **A)** 1 µg/L (χ^2_1=3.1, p=0.079), 10 µg/L (χ^2_1=11.6, p≤0.001) and 50 µg/L (χ^2_1=10.5, p≤0.001) of imidacloprid (IM), **B)** 1 µg/L (χ^2_1=11.6, p≤0.001), 10 µg/L (χ^2_1=20.0, p≤0.001) and 50 µg/L (χ^2_1=9.5, p=0.002) clothianidin (CLO), **C)** 1 µg/L (χ^2_1=9.5, p=0.002), 10 µg/L (χ^2_1=21.0, p≤0.001) and 50 µg/L (χ^2_1=21.0, p≤0.001) thiamethoxam (TMX) and **D)** 1 µg/L (χ^2_1=6.3, p=0.012), 10 µg/L (χ^2_1=3.1, p=0.079) and 50 µg/L (χ^2_1=3.1, p=0.079) thiacloprid (TCL). n=27-32 flies per treatment.

3.4.3 Neonicotinoids affected activity levels under light:dark conditions

Many of the neonicotinoids also influenced the total activity levels of flies during LD, tending to cause an increase in activity (Fig. 3.9). Imidacloprid (Fig. 3.9A) caused an increase in night-time activity at 1 µg/L, as did clothianidin (Fig. 3.9B) for every dose tested. Clothianidin also caused a small decrease in daytime activity at 1 µg/L, whilst thiacloprid (Fig.3.9D) caused an increase in daytime activity for every dose tested. Thiamethoxam had no effect (Fig. 3.9C).

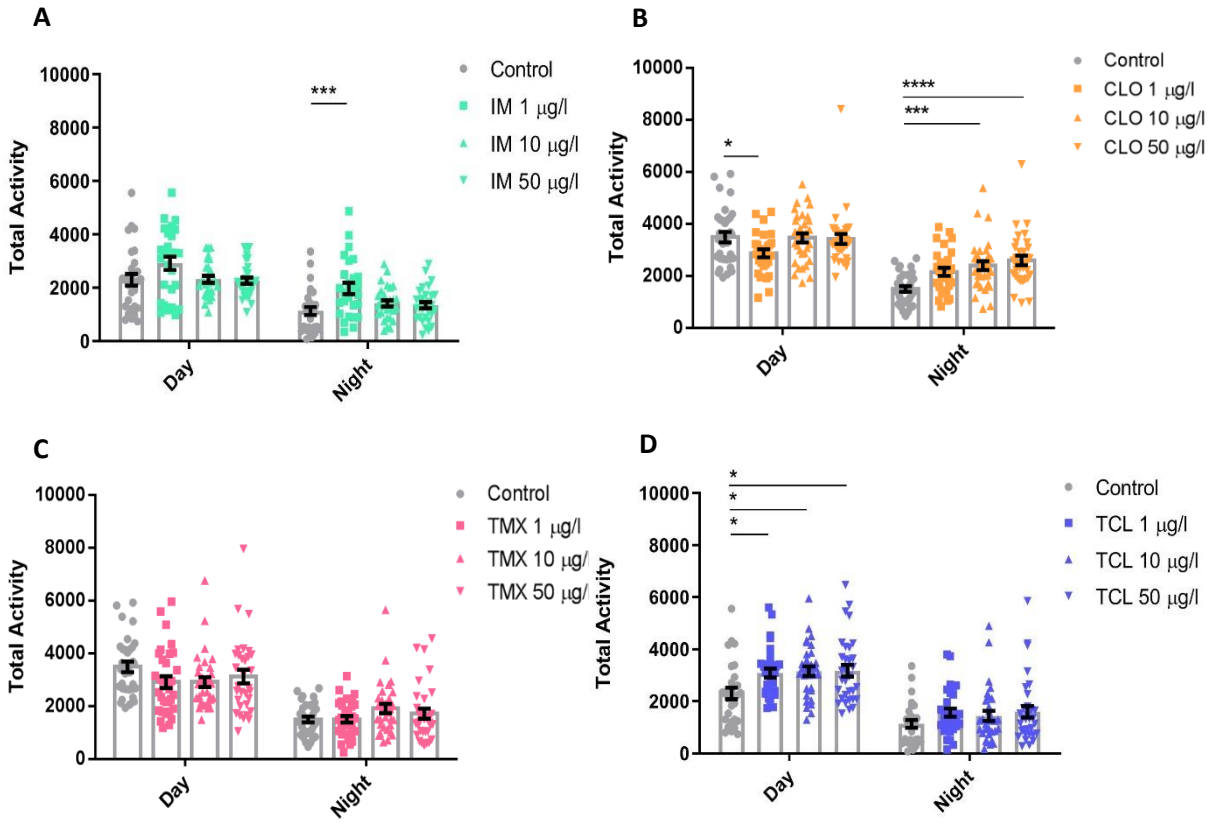

Figure 3.9: Neonicotinoids effect activity levels under light:dark conditions

Total activity counts for the day and night for flies exposed to 1, 10 or 50 µg/L of **A**) imidacloprid (IM), for the day ($F_{3,114}$=2.7, p=0.051) and night ($F_{3,114}$=5.4, p=0.002), **B**) clothianidin (CLO), day ($F_{3,120}$=2.9, p=0.036) and night ($F_{3,120}$=8.8, p≤0.001), **C**) thiamethoxam (TMX), day ($F_{3,124}$=1.6, p=0.199) and night ($F_{3,124}$=1.7, p=0.176) or **D**) thiacloprid (TCL), day ($F_{3,119}$=4.1, p=0.008) and night ($F_{3,119}$=1.3, p=0.290).Each data point represents a single fly, n=28-32 flies per treatment.

3.4.4 Neonicotinoids affected period length under light:dark conditions

The period length in LD (Fig. 3.10) was affected by both clothianidin (Fig. 3.10B) and thiacloprid (Fig. 3.10D) but the effect size was negligible.

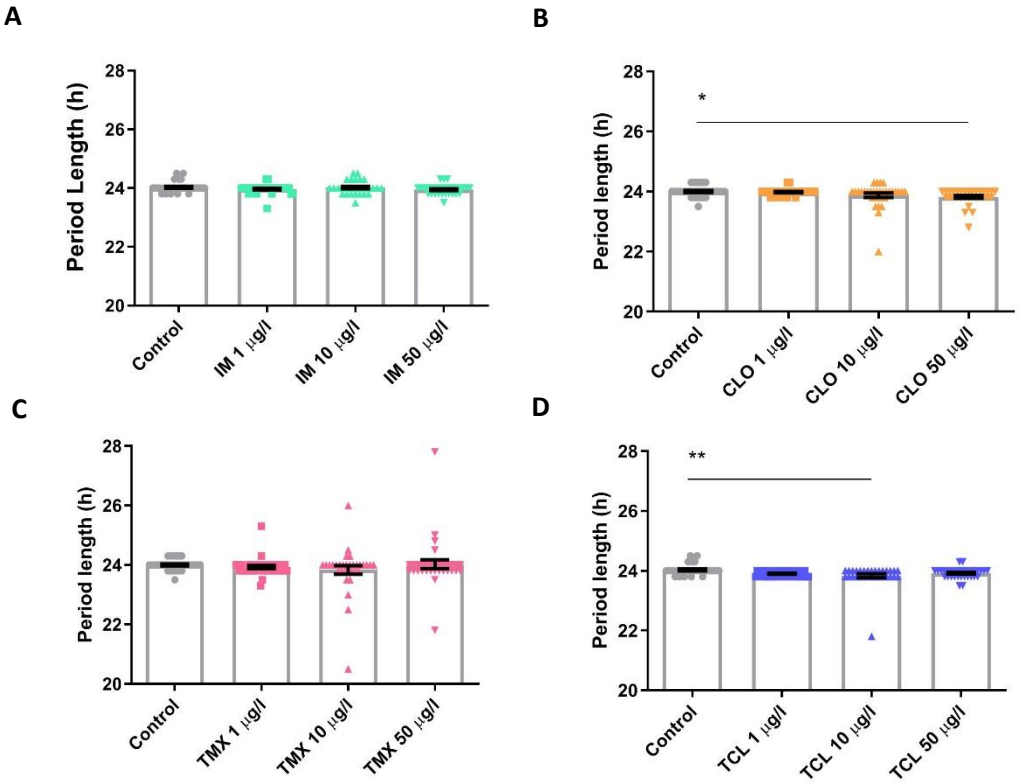

Figure 3.10: Neonicotinoids can affect period length in light:dark conditions

Mean period length for flies exposed to 1, 10 or 50 µg/L of **A)** imidacloprid (IM) ($F_{3,114}$=1.4, p=0.239, **B)** clothianidin (CLO) ($F_{3,120}$=3.09, p=0.0296), **C)** thiamethoxam (TMX) ($F_{3,124}$=0.6, p=0.590) and **D)** thiacloprid (TCL) ($F_{3,119}$=3.57, p=0.0163). Each data point represents a single fly, n=28-32 flies per treatment.

3.5 Neonicotinoids affected sleep quantity and quality

Sleep is a behaviour partially controlled by the clock, as well as receiving input from the sleep homeostat[124]. Neonicotinoids caused a significant disruption to both the quantity and quality of sleep in flies.

3.5.1 Neonicotinoids reduced total sleep

All of the neonicotinoids tested had an effect on the total quantity of sleep achieved by flies (Fig 3.11). For the three banned neonicotinoids this effect was observable during night-time sleep. The greatest effect occurred in flies exposed to clothianidin (Fig. 3.11B), with every dose tested (1-50µg/L) causing a reduction in total night-time sleep. A dose of 50µg/L of thiamethoxam (Fig. 3.11C)

or 10µg/L of imidacloprid (Fig. 3.11A) also caused a reduction in night-time sleep. Thiacloprid (Fig. 3.11D) on the other hand, affected daytime sleep, causing a reduction at every dose tested (1-50µg/L).

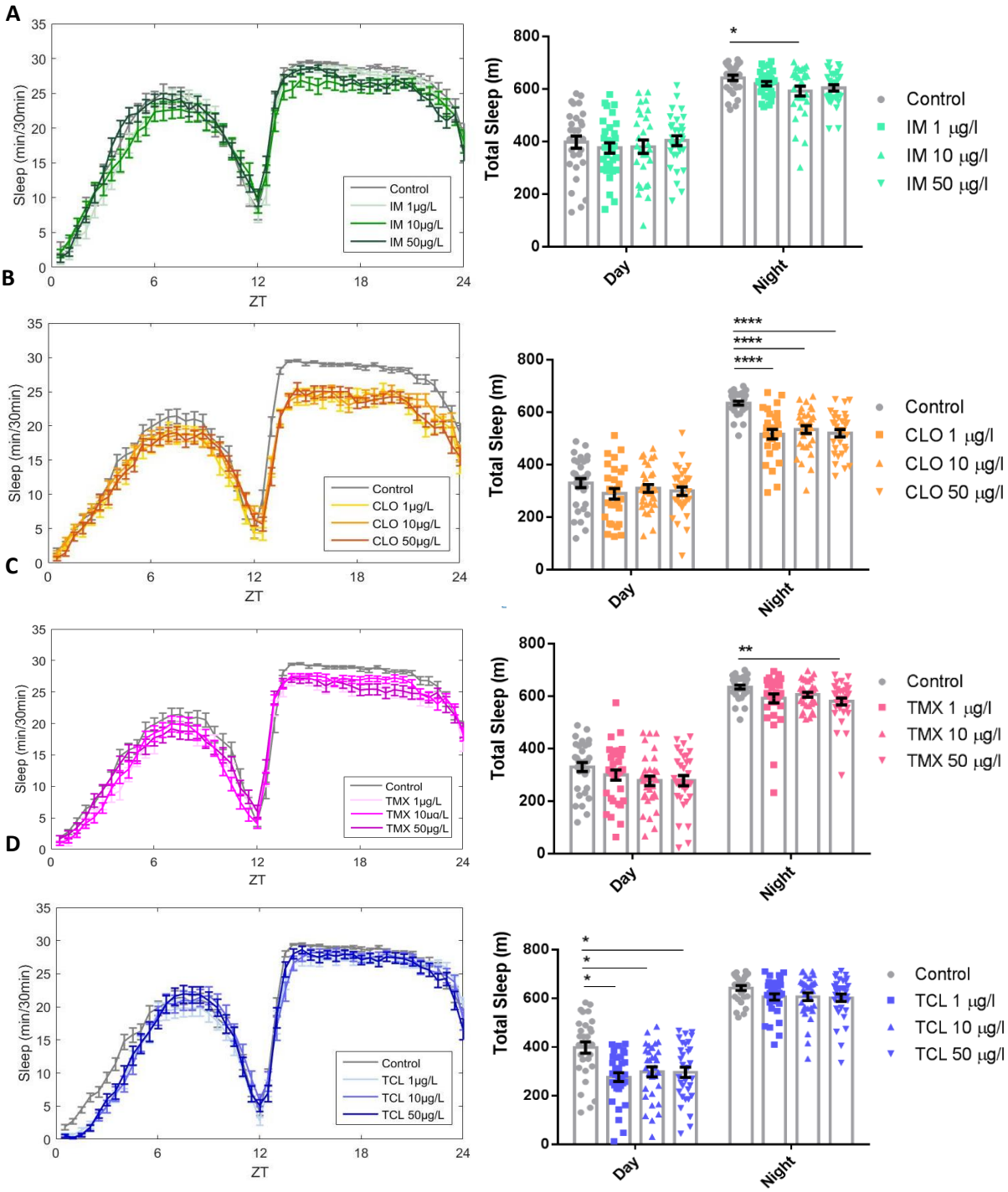

Figure 3.11: Neonicotinoids effect total sleep quantity achieved

Total sleep achieved over the day and night (lights on = ZT 0-12, lights off = ZT 12-24) for flies exposed to 1, 10 or 50 µg/L of **A**) imidacloprid (IM), day ($F_{3,114}$=0.4, p=0.772) and night ($F_{3,114}$=2.9, p=0.040), **B**) clothianidin (CLO), day ($F_{3,120}$=1.0, p=0.378) and night ($F_{3,120}$)=16.6, p≤0.001), **C**) thiamethoxam (TMX), day ($F_{3,124}$=1.8, p=0.154) and night ($F_{3,124}$=3.7, p=0.013) or **D**) thiacloprid (TCL), day ($F_{3,120}$=4.5, p=0.005) and night ($F_{3,120}$=1.8, p=0.157).

3.5.2 Neonicotinoids caused fragmentation of sleep

By assessing the quantity and length of sleep episodes that comprised sleep, it was possible to visualise sleep structure and make judgements about the quality of sleep that might be achieved and the mechanisms that may be experiencing disruption. The three banned neonicotinoids caused a fragmentation of sleep, with sleep being composed of more episodes (Fig. 3.12) of a shorter length (Fig. 3.13). This effect was most extreme for clothianidin-exposed flies. At every dose tested, clothianidin caused an increase in the number of sleep episodes (Fig. 3.12B) achieved and a reduction in the mean length of these episodes (Fig 3.13B) for both day and night. Thiamethoxam exposed flies exhibited a similar effect size for both measures but only during night-time sleep, with flies experiencing a greater number of sleep episodes (Fig. 3.12C) of shorter length (Fig. 3.13C). Imidacloprid too caused an increase in the number of sleep episodes (Fig. 3.12A) and a reduction in episode length (Fig. 3.13A) during night time sleep however this effect was only significant for doses of 10 and 50 µg/L. Thiacloprid caused an increase in the number of sleep episodes achieved during the night at 10 µg/L (Fig 3.12D). Thiacloprid also caused a reduction in mean episode length (fig. 3.13D) but unlike the banned neonicotinoids, this effect was only visible for daytime sleep.

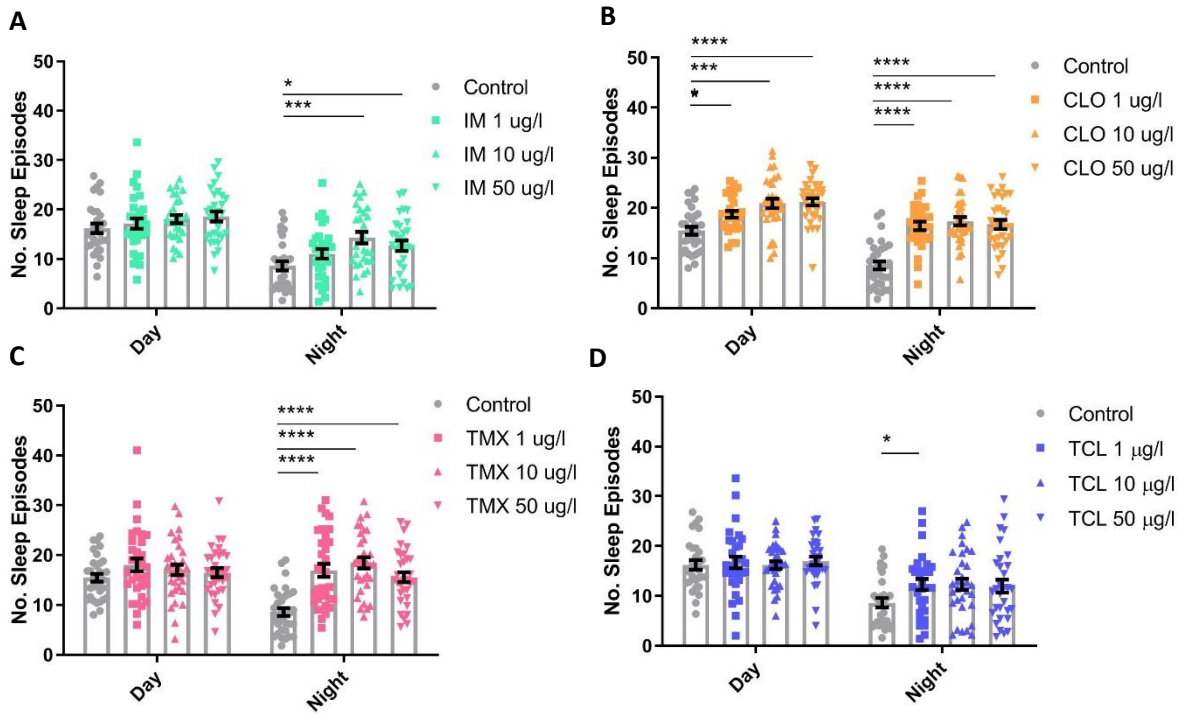

Figure 3.12: Neonicotinoids effected the number of sleep episodes initiated

The number of sleep episodes initiated during the day and night for flies exposed to 1, 10 or 50 µg/L of **A**) imidacloprid (IM), day ($F_{3,114}$=1.2, p=0.320) and night ($F_{3,114}$=5.5, p=0.001), **B**) clothianidin (CLO), day ($F_{3,120}$=11.5, p≤0.001) and night ($F_{3,120}$=25.0, p≤0.001), **C**) thiamethoxam (TMX), day ($F_{3,124}$=1.1, p=0.344) and night ($F_{3,124}$=17.0, p≤0.001) or **D**) thiacloprid (TCL), day ($F_{3,120}$=0.2, p=0.872) and night ($F_{3,120}$=3.0, p=0.034). Each data point represents a single fly, n=28-32 flies per treatment.

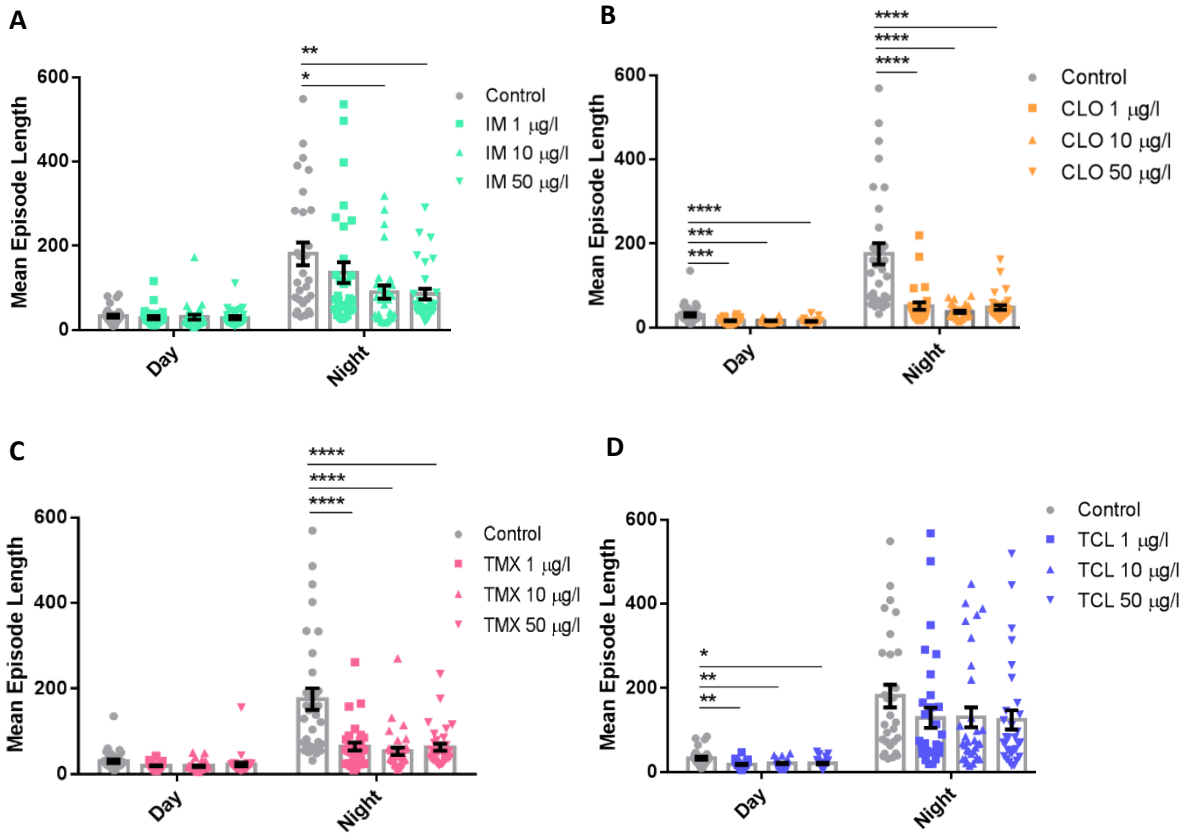

Figure 3.13: Neonicotinoids affected the mean length of sleep episodes

The mean length of sleep episodes initiated during the day and night for flies exposed to 1, 10 or 50 µg/L of **A**) imidacloprid (IM), day ($F_{3,114}$=0.2, p=0.889) and night ($F_{3,114}$=4.5, p=0.005), **B**) clothianidin (CLO), day ($F_{3,120}$=9.9, p≤0.001) and night ($F_{3,120}$=21.8, p≤0.001), **C**) thiamethoxam (TMX), day ($F_{3,124}$=2.5, p=0.061) and night ($F_{3,124}$=15.7, p≤0.001) or **D**) thiacloprid (TCL), day ($F_{3,120}$=5.2, p=0.002) and night ($F_{3,120}$=2.0, p=0.121). Each data point represents a single fly, n=28-32 flies per treatment.

3.5.3 Neonicotinoids can increase sleep latency

Sleep latency (Fig. 3.14) is a measure of how long it takes an individual to achieve sleep after a change in conditions (e.g. lights on or lights off). Both imidacloprid and clothianidin had no effect on this behaviour (Fig. 3.14A-B). Thiamethoxam showed a very small decrease in sleep latency after lights off for 10 µg/L (Fig. 3.14C), however thiacloprid showed the greatest effect, with every dose tested causing an increase in latency after lights on and similarly for lights off at a dose of 50 µg/L (Fig. 3.14D).

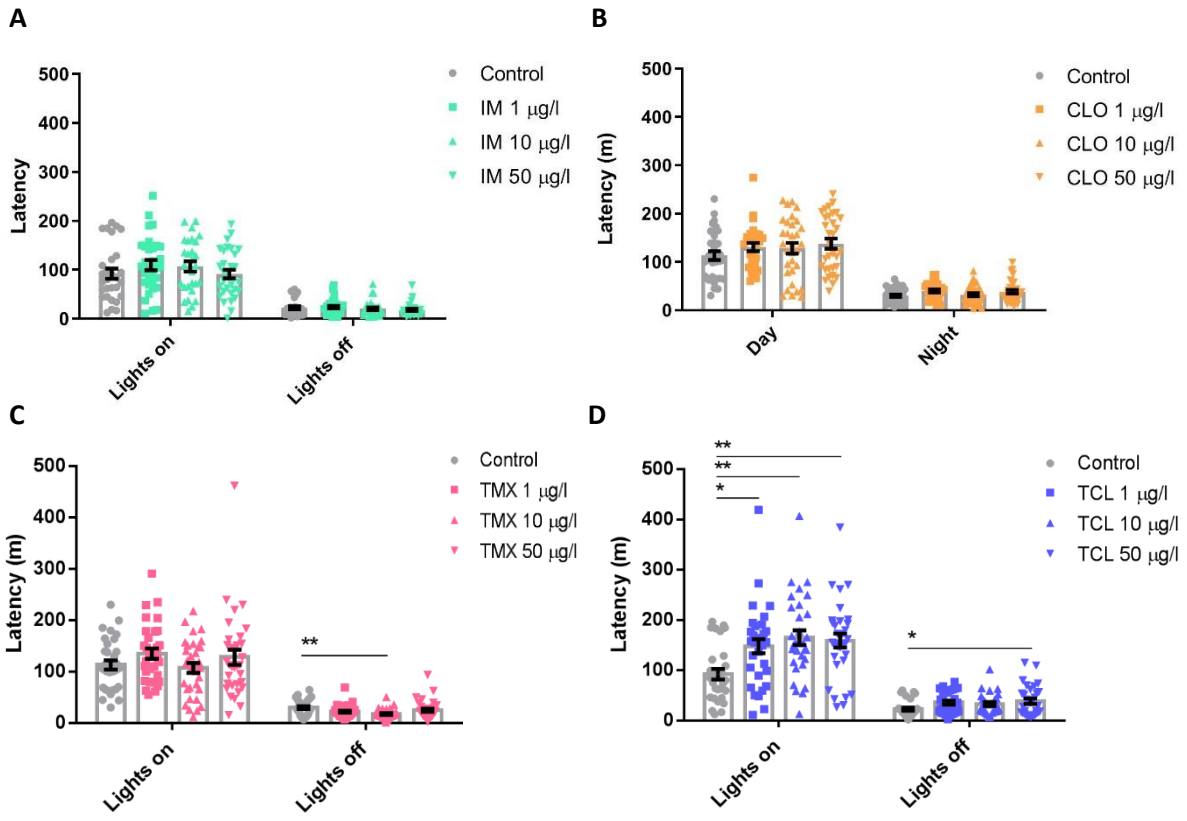

Figure 3.14: Neonicotinoids affected sleep latency in flies

The mean latency (min) before sleep was initiated after lights on or lights off for flies exposed to 1, 10 or 50 µg/L of **A**) imidacloprid (IM), day ($F_{3,114}$=0.9, p=0.441) and night ($F_{3,114}$=0.9, p=0.468), **B**) clothianidin (CLO), day ($F_{3,120}$=1.1, p=0.333) and night ($F_{3,120}$=2.0, p=0.124), **C**) thiamethoxam (TMX), day ($F_{3,124}$=1.3, p=0.264) and night ($F_{3,124}$=4.3, p=0.007) or **D**) thiacloprid (TCL), day ($F_{3,120}$=5.6, p=0.001) and night ($F_{3,120}$=2.7, p=0.50). Each datapoint represents a single fly, n=28-32 flies per treatment.

3.6 Discussion

The findings in this chapter clearly indicate that neonicotinoid pesticides can reduce behavioural rhythmicity and disrupt normal sleep behaviour. Circadian rhythmicity and sleep have received a large amount of research attention in *Drosophila*, as have the sub-lethal behavioural effects of neonicotinoid pesticides. However, this appears to be the first time that the effects of neonicotinoids on circadian outputs and sleep has been thoroughly investigated. This section comprises a discussion of the findings described in Chapter 3. For a discussion of how these findings relate to the other chapters in this thesis and to the research question as a whole, please refer to Chapter 6.

3.6.1 The banned neonicotinoids showed differing effects to thiacloprid

In this chapter, I tested the effects of three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) covered by the European Union's (EU) neonicotinoid ban. I also tested thiacloprid, one of the two remaining neonicotinoids approved for use in the EU. Thiacloprid is classified by the EU as candidate for substitution, meaning that the EU suggests alternative methods for pest control be assessed in the hope of replacing its use. The current status of thiacloprid as an approved substance in the EU is up for debate at the end of 2019 and due for renewal in April 2020, making the collection of evidence about its potential off target effects important and timely.

The basis for the ban was the risks posed to pollinators by the continued agricultural use of these three neonicotinoids. The scientific community had previously published a large quantity of evidence identifying the harmful sublethal effects of these pesticides[265,266]. The majority of this research has focused on the effects of the three neonicotinoids covered by the ban but comparative studies on the effect size of thiacloprid compared to these has indicated that thiacloprid is often less toxic. For example thiacloprid has a higher lethal dose than the other three neonicotinoids[265] and comparatively much higher doses are required to cause the same behavioural effects[267,268]. This is reflected by other work carried out in our lab (Appendix 1) finding that of the four neonicotinoids tested, thiacloprid was the least toxic when measuring effects on longevity, offspring survival and learning and memory in flies.

This is not to claim that thiacloprid is safe for use. Many of the results mentioned above show that thiacloprid has lethal and sublethal effects. However, this does suggest that the results from Chapter 3, in which thiacloprid consistently caused less disruption to behavioural rhythmicity than the other three banned neonicotinoids, is in line with previous work. The key difference between thiacloprid and the banned neonicotinoids is the molecular makeup of the compounds; the three banned

neonicotinoids are all N-nitroguanidine neonicotinoids whereas thiacloprid is an N-cyanoamidine neonicotinoid[33]. Breakdown of toxic substances within the insect body is performed by enzymes in the cytochrome P-450 family[269], and neonicotinoid-resistant pests found in the field often show mutations resulting in over expression of these enzymes[77,270]. Studies have identified a cytochrome P450 enzyme in honey bees and solitary bees that can efficiently metabolise thiacloprid but has little efficacy against imidacloprid or the other banned neonicotinoids[83,271]. This may explain the lower toxicity of thiacloprid and its lesser impact on behaviour compared to the banned neonicotinoids.

Of the banned neonicotinoids, I found that clothianidin and thiamethoxam caused greater disturbances to behaviour than imidacloprid. Imidacloprid appears to be marginally less lethal than clothianidin and thiamethoxam[265]. A possible explanation for this is the difference in their efficacy as agonists. A study using whole-cell patch clamp in the CNS of *Drosophila* larvae found that imidacloprid is a partial agonist of the nAChRs, causing a maximal current amplitude of 10-14% of that observed for ACh, the natural agonist[272]. On the other hand, clothianidin appears to be a super-agonist, evoking a maximal current amplitude 56% greater than that recorded for ACh. Research using cockroach neurons suggests that this difference in efficacy may be due to a difference in the structure of the N-nitroimine group, which is cyclic in imidacloprid but not in clothianidin[24].

Thiamethoxam is a pro-drug for clothianidin[273], being metabolised into clothianidin in the body, explaining its high efficacy. Perhaps unexpectedly, thiamethoxam appeared to have a greater impact than clothianidin on behavioural rhythmicity. As well as clothianidin, thiamethoxam forms another toxic metabolite, *N*-desmethyl thiamethoxam, which has a lethality similar to that of imidacloprid[273]. Potentially, combined exposure to these two toxic metabolites explains the greater effect observed for thiamethoxam than either imidacloprid or clothianidin in some assays.

3.6.2 Neonicotinoids affect locomotor function and activity levels

The first behaviour tested was locomotor function, as previous work in bees has found that neonicotinoids can affect mobility in a variety of ways, ranging from hyperactivity to immobility[274,275]. A study carried out in cockroaches found that neonicotinoids can cause excitation of neurons from the three thoracic ganglia, which are involved in motor function[24] suggesting neonicotinoids may be able to directly affect locomotion and motor skills.

The effect of four neonicotinoids on locomotion was tested by utilising the flies innate negative geotaxis. All three of the banned neonicotinoids reduced the capacity of flies to carry out this innate climbing behaviour in a dose dependant way. This result mirrors a study carried out in honeybees on phototaxis[276] (innate movement towards light), a somewhat similar behaviour. Exposure to

thiamethoxam resulted in a reduced capacity to climb up a vertical surface towards light. Individuals showed hyperactivity but impaired motor functions, preventing many of them from ascending[276]. This duality, where neonicotinoids appear to cause an increase in basic locomotion whilst also reducing the capacity of insects to perform more complex locomotive tasks appears repeatedly in the literature[275-280]. Neonicotinoid exposure is often found to increase distance and speed travelled on foot by bees, with decreased rests[275-277]. However exposure has also been shown to reduce bee's capacity to carry out more demanding activities such as flight[278], climbing[276] and postural control[275]. This loss in complex motor function has been identified in other species too, for example reduced capacity for web building[279] and ballooning (creation of web 'balloons' to allow wind transportation)[280] in spiders.

The results shown in this chapter also display this duality in *Drosophila*. Whilst neonicotinoid exposure caused a significant reduction in climbing ability in flies, the baseline activity level tended to increase. All four neonicotinoids tested had an effect on activity levels. In general, neonicotinoids caused an increase in activity, mirroring the hyperactivity observed in other insects[275]. The only dose to cause a decrease in activity was 50 µg/L of imidacloprid. A study comparing the effects of imidacloprid and thiamethoxam on locomotion in bees found that imidacloprid reduced locomotion where thiamethoxam greatly increased it[277]. This could be due to the difference observed in the efficacy of imidacloprid and clothianidin (for which thiamethoxam is a precursor) as agonists. Remember that clothianidin causes a greater response than ACh, the natural agonist whereas imidacloprid causes a response lower than ACh. If the neonicotinoids are outcompeting the natural agonist, this difference in efficacy could explain why clothianidin and thiamethoxam cause an increase in activity compared to control flies whilst imidacloprid causes a decrease in activity. Thus, the effects for neonicotinoids on activity and locomotor ability in *Drosophila* appear to match published findings for other insects and suggest that behavioural rhythmicity will not be limited by neonicotinoid induced immobility.

3.6.3 Neonicotinoids reduce behavioural rhythmicity in constant conditions

The three banned neonicotinoids caused a clear breakdown in rhythmicity after the *zeitgeber*, light, was removed. Neonicotinoid exposure under constant conditions caused a decrease in the mean rhythmicity statistic to approximately 1.5, the boundary of total arrhythmia. This is reflected in the large proportion of exposed populations who exhibited behavioural arrhythmicity. As covered in Chapter 1, the ability to maintain rhythmicity without *zeitgeber*s is reliant on an internal or endogenous clock. In *Drosophila* this endogenous clock is reliant on the s-LNvs pacemaker neurons[151]. The rapid breakdown of rhythmicity observed in constant conditions suggests that

neonicotinoid exposure may be interfering with the s-LNvs. The s-LNvs are nicotinic so neonicotinoids, as agonists of the nAChRs, could act upon these neurons, causing excitation and or a depolarisation block. The main source of ACh into the s-LNvs is proposed to be the Hofbauer-Buchner eyelet (H-B eyelet). These light sensing organs have ACh expressing terminals that come into direct contact with the s-LNvs[90,281] and excitation of the H-B eyelet leads to a cAMP increase in the s-LNvs[156]. This provides a hypothetical route for neonicotinoids to disrupt the electrical state of the s-LNvs, through excitation and/or a depolarisation block, potentially acting as a sort of light signal.

The electrical activity of the s-LNvs is integral to their function as pacemakers, allowing them to communicate with downstream neurons to drive the clock and influence circadian outputs such as behavioural rhythmicity. The electrical activity of the pacemaker neurons also appears to be important for the continued oscillation of the molecular clock in DD, with electrical silencing of the PDF+ LNvs causing rundown of the molecular clock under constant conditions[167].

Previous research using flies that overexpressed ion channels in the PDF+ pacemaker neurons resulted in either hyperexcitation[165] or electrical silencing[151,167] depending whether the channel was an Na^+ or K^+ channel, with either resulting in disrupted behavioural rhythmicity. Thus, it is possible that neonicotinoids are causing the reduction in rhythmicity observed in this chapter though agonistic action at the s-LNvs, disrupting the membrane clock and potentially also the molecular clock.

3.6.4 Neonicotinoids cause a reduction in rhythmicity strength in light:dark conditions

Both imidacloprid and thiamethoxam also caused a significant reduction in the mean rhythmicity of *Drosophila* in LD conditions and all four of the neonicotinoids tested caused an increase in the proportion of the population that was arrhythmic; up to 19% in some cases. Whilst in each case the majority of flies remained strongly rhythmic, the reduction in rhythm strength compared to control flies suggests that the neonicotinoids are interfering with the clock in LD conditions too.

As described in Chapter 1, both the s-LNvs and the l-LNvs are sufficient to drive rhythmicity in LD conditions[282,283]. Like the s-LNvs, the l-LNvs are also nicotinic, with ACh input coming from the visual circuitry such as the L2 monopolar neurons from the lamina[91]. Acetylcholine is also utilised by the l-LNvs for maintaining synchronicity in firing rate between the two hemispheres[92]. Agonistic action by neonicotinoids at either set of LNvs could cause a disruption to the output of the clock or a loss of synchronicity that could weaken behavioural rhythmicity. Hyperexcitation of the l-LNvs prevents them from responding to light through changes in firing rate or membrane potential[153]. Possibly the

neonicotinoids are acting at the cholinergic synapses between the visual circuitry and the LNvs, causing excitation and or depolarisation during both day and night and weakening the strength of the signal from the light sensing organs, resulting in weakened behavioural rhythmicity. A study looking at the effect that electrically silencing the PDF+ LNvs had on circadian outputs in LD found that, despite having a functional molecular clock in the LNvs, the behavioural rhythmicity of these flies in LD was impaired due to the loss of day-night differences in electrical activity. In this case, as with neonicotinoid exposed flies, the effects on behavioural rhythmicity were much more severe in constant conditions than in LD[167]. Behavioural rhythmicity in LD is likely supported by cryptochrome (CRY), a cell-autonomous photoreceptor which is part of the molecular clock and is sufficient to set the pace of behavioural rhythmicity in the absence of eyes and other light sensing organs[158].

The effects of neonicotinoids on the rhythm strength of flies in LD conditions suggest that neonicotinoids could have disruptive effects on the behavioural rhythmicity of insects in the field. A criticism of constant conditions experiments can be that, though they elucidate effects on the endogenous clock of individual animals, this will not necessarily translate into harm in the field, under natural light conditions. Of course, a weakened endogenous clock can have many physiological consequences as the clock is involved in the expression of hundreds of genes[95] and the synchronisation of many physiological functions[96]. Mutant flies such as *per^{01}* who possess no functional internal clock can exhibit diurnal behaviour in LD conditions[284] but have reduced lifespans, mobility impairments and early-onset neurodegeneration[96]. However, the data presented in this chapter shows that neonicotinoids also weaken the clock in the presence of strong environmental cues, suggesting that they may also have direct effects on behavioural rhythmicity in the field.

3.6.5 Neonicotinoids have little effect on period length

None of the neonicotinoids tested had a notable effect on period length. Given that the neonicotinoids appear to cause a breakdown of behavioural rhythmicity, it is unsurprising that a new behavioural rhythm with a divergent period length does not emerge.

3.6.6 Neonicotinoids reduce and fragment sleep

Sleep behaviour appears to be more susceptible to neonicotinoid exposure than rhythmicity; each of the neonicotinoids tested influenced the quantity and quality of sleep achieved in flies. For the three banned neonicotinoids, there was a reduction of total night-time sleep. This reduction was the result of a change in the structure of sleep behaviour. Sleep became highly fragmented, comprising frequent, much shorter sleep episodes. These neonicotinoids appear to interfere with the maintenance of sleep, causing waking or arousal early on in the sleep episode.

As mentioned in the discussion of the rhythmicity data above (section 3.6.4), the l-LNvs are nicotinic[91] and present a potential target *via* which the neonicotinoids may be disrupting circadian output of the clock. As well as their role in the clock, the l-LNvs are a vital part of the sleep/wake circuitry, acting as key arousal neurons. Excitation of these neurons by neonicotinoids could explain why exposed flies appear to struggle to maintain sleep. Previous work which focused on the l-LNvs' role as arousal neurons found that hyperexcitation of the l-LNvs caused sleep behaviour similar to that seen in neonicotinoid exposed flies; reduction in night-time sleep and shorter sleep episodes[153].

Interestingly, neonicotinoid exposure did not increase the sleep latency, suggesting that despite their difficulty maintaining sleep, these flies had a functional sleep homeostat. This is supported by the increased number of sleep episodes initiated in response to the decrease in episode length; possibly an attempt to counteract the loss of sleep. Whilst arousal and sleep timing are dictated by the l-LNvs, the sleep homeostat is located elsewhere, such as the R2 ring neurons of the ellipsoid body, which are mediated by the neurotransmitter N-Methyl-d-aspartic acid (NMDA) glutamate receptor signalling[190]. This could explain why wakefulness but not the sleep homeostat appears to be disrupted by neonicotinoid exposure.

Thiacloprid caused a different change in sleep behaviour to the other neonicotinoids. The main effect of thiacloprid occurred during daytime, which is when flies exhibited a significant reduction in sleep. This was partially due to a slight decrease in sleep episode length (although the effect size was very small) but was mainly due a large increase in sleep latency after lights on. This is mirrored by the greatly increased daytime activity levels in thiacloprid exposed flies. As shown earlier in this chapter (section 3.3.1) neonicotinoids tend to increase activity levels. Thiacloprid was the only neonicotinoid not to affect rhythmicity strength so potentially a functional clock is allowing thiacloprid exposed flies to contain this hyperactivity within the daytime, when it is more physiologically relevant, resulting in a significant reduction in daytime, but not night-time, sleep. Alternatively, flies exposed to thiacloprid may have largely metabolised the drug by lights off, leading to very little effect on behaviour during the night. In *Drosophila*, most eating occurs during the first two hours after lights on[285], and as discussed above thiacloprid is the most rapidly metabolised of the neonicotinoids tested. The first two hours after lights on is also the section of the day for which thiacloprid exposed flies experience the majority of sleep reduction.

Exposure to any of the four neonicotinoids tested caused a reduction in total sleep. These findings could have concerning repercussions for insects in the field. Sleep has been proven to play an important role in memory consolidation, for example sleep is important for both odour associated learning[106] and navigational memory formation in honeybees[16]. In relation to this, it is thought that

sleep also allows synaptic-downscaling[104]; a scaled reduction in the strength of all synaptic connections, allowing the elimination of weak synapses and leading to an increased signal:noise ratio in the brain[180]. Potential examples of this have been identified in *Drosophila*[179]. As reduced sleep can reduce odour-based learning and navigation in bees[16,106], reductions in total sleep such as observed in neonicotinoid exposed *Drosophila* may reduce their ability to forage and pollinate effectively. Neonicotinoid induced learning and memory deficits have already been identified in honeybees[36,286], bumblebees[287] and *Drosophila* (Appendix 1), and sleep deprivation could be a component of this.

3.7 Concluding Remarks:

- Neonicotinoid exposure tended to decrease locomotor ability but increase activity.

- The neonicotinoids imidacloprid, clothianidin and thiamethoxam all reduce behavioural rhythmicity.

- Imidacloprid, clothianidin, thiamethoxam and thiacloprid all cause fragmentation of sleep, leading to a greater number of sleep episodes that are shorter in length and resulting in a reduction in total sleep.

4

Chapter 4: *RNAi* mediated knockdown of nAChR subunits Dα1, Dα3 and Dβ2 disrupts circadian rhythmicity and sleep

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4.1 Introduction

This chapter aims to determine the mechanism of action through which neonicotinoids cause the disruptions to circadian rhythmicity and sleep behaviour observed in Chapter 3. Through the *RNAi* mediated knock down of neonicotinoid susceptible nAChR subunits in the clock neurons, the role of these subunits in normal sleep and circadian behaviour was identified. These knock down flies were then additionally exposed to neonicotinoids, to investigate whether the neonicotinoids could be acting directly *via* the clock neurons and whether the subunits investigated where mediating these effects. For these experiments, the subunits Dα1, Dα3 and Dβ2 were investigated. Dα1 and Dβ2 have been shown to mediate neonicotinoid susceptibility in *Drosophila*[84] and Dα3 is rhythmically expressed in the LNvs and is a candidate for *rye,* which is an nAChR involved in sleep behaviour, suggesting it could play a role in the clock[86,288]. Thus, it was thought that these subunits may participate in the nAChRs involved in the effects of neonicotinoids on the clock and sleep.

The circadian plasticity of the s-LNv dorsal terminals was also explored. The day/night differences in branching and PDF accumulation in these terminals appear to be heavily influenced by the electrical state of the LNvs[147,151,165]. This circadian remodelling is important for producing behavioural rhythmicity and may also influence sleep behaviour[151,167,193,289]. Thus, the effect of neonicotinoids and neonicotinoid susceptible nAChR subunit knockdowns on this plasticity and PDF accumulation was explored.

Section 4.2 covers the effects of the knock down of Dα1, Dα3 or Dβ2 in the clock neurons on circadian rhythmicity and sleep in flies. Also covered in this section, is the effect of imidacloprid or clothianidin exposure on the clock and sleep in these knock down flies. Section 4.3 shows the effects of imidacloprid or clothianidin exposure on circadian plasticity and PDF cycling in the s-LNv dorsal terminals. Section 4.4 illustrates the effects that the knock down of either Dα1 or Dβ2 in the LNvs

has on the branching and PDF cycling of these terminals. Section 4.5 discusses the findings of this chapter and Section 4.6 summarises these findings.

4.2 *RNAi* mediated knock down of Dα1, Dα3 and Dβ2 in the clock disrupted rhythmicity and sleep behaviour

Knock down of Dα1, Dα3 and Dβ2 nAChR subunits in the clock neurons caused significant changes to circadian and sleep behaviour. These changes were similar for each subunit knock down and resembled those seen for neonicotinoid exposure in Chapter 3. Knock down flies all experienced a reduction in rhythmicity and fragmentation of sleep.

4.2.1 *RNAi* mediated knock down of Dα1 in the clock reduced rhythmicity and disrupted sleep

The knock down of Dα1 in the clock neurons caused a reduction in mean rhythmicity strength compared to that of the *GAL4* alone control (*tim/+*) flies (Fig. 4.1A,C). The proportion of flies that were arrhythmic was highest for the knock down flies, with 44% of the population exhibiting arrhythmicity. This was followed closely by the *UAS* alone control (*Dα1 RNAi /+*), in which 38% of the flies were arrhythmic, contrasting with the *tim/+* flies, in which all flies were rhythmic (Fig. 4.1B). The reduction in rhythmicity observed for the *Dα1 RNAi /+* suggest that the *UAS* may be leaky and could be expressed more widely. The *Dα1 RNAi /+* control flies were more active than *tim/+* flies in both day and night, with the activity of knockdown flies falling between the two controls (Fig.4.1D).

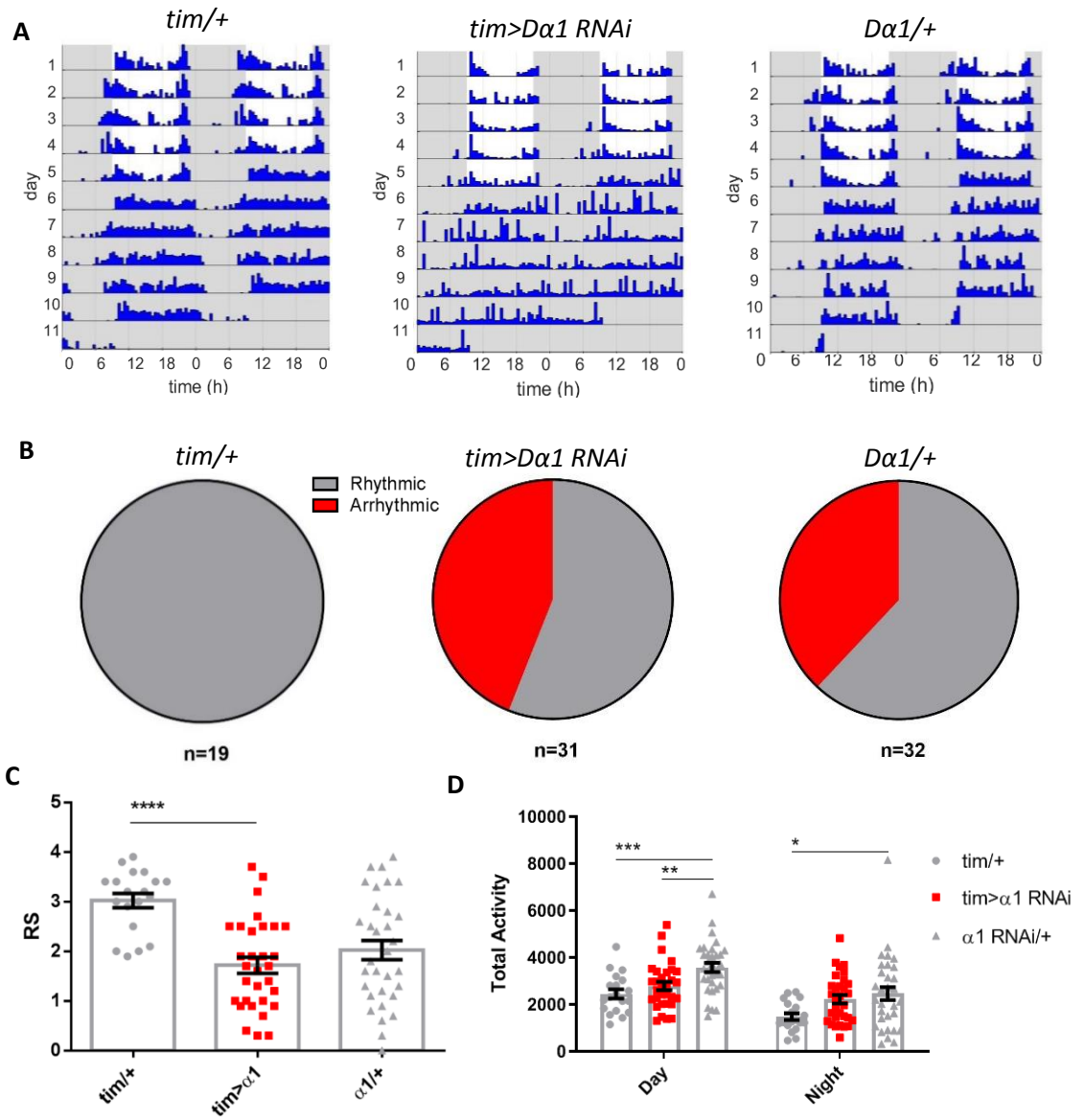

Figure 4.1 Knock down of Dα1 in the clock reduced rhythmicity

A) Representative actograms for *tim/+*, *tim>Dα1 RNAi* and *Dα1 RNAi/+*. **B)** Proportion of flies that were arrhythmic for *tim>Dα1 RNAi*, compared to *tim/+* (χ^2_1=55.4, p≤0.001), and *Dα1 RNAi/+* (χ^2_1=1.7, p=0.19). **C)** Mean behavioural rhythmicity for each genotype ($F_{2,79}$=11.8, p≤0.001). **D)** Total daily activity for each genotype during the day ($F_{2,79}$=8.7, p≤0.001) and the night ($F_{2,79}$=4.0, p=0.022). Each data point in the histogram represents a single fly, n=19-32 flies per treatment.

The knock down of Dα1 changed the shape of daily sleep (Fig. 4.2A) but did not affect the total quantity of sleep achieved compared to control genotype flies (Fig. 4.2B). However, the knock down did affect the structure of sleep, causing fragmentation. The *tim>α1 RNAi* flies initiated more sleep episodes during night-time sleep than either of the control groups (Fig. 4.2C). They also had shorter sleep episodes than the control *tim/+*, during both the day and night (Fig. 4.2D).

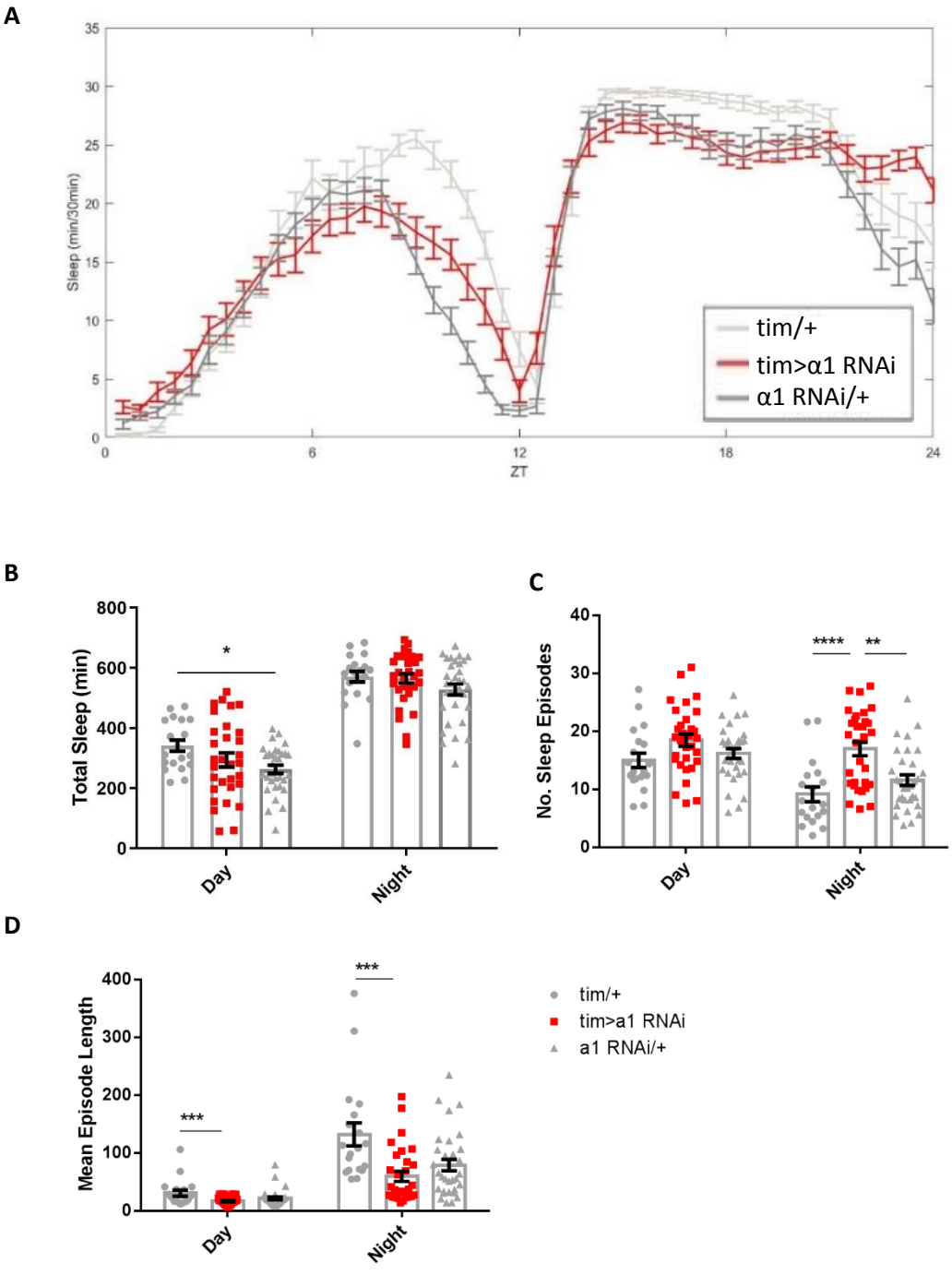

Figure 4.2 Knock down of Dα1 in the clock disrupted sleep

A) Average sleep achieved by each genotype over the 24 hour period. **B)** Total amount of sleep achieved by each genotype during the day ($F_{2,79}$=3.5, p=0.034) and the night ($F_{2,79}$=1.8, p=0.174). **C)** number of sleep episodes initiated for each genotype in the day ($F_{2,79}$=2.9), p=0.063 and night ($F_{2,79}$=12.3, p≥0.001) and **D)** mean sleep episode length in day ($F_{2,79}$=5.1, p=0.008) and night ($F_{2,79}$=8.3, p=0.001). For the histograms, each data point represents a single fly, n=19-32 flies for each genotype.

4.2.2 Neonicotinoid exposure in Dα1 knockdown flies disrupted sleep but not rhythmicity

The exposure of *tim>α1 RNAi* flies to 50 µg/L of either imidacloprid or clothianidin had no additional effect on mean rhythmicity (Fig.4.3A,C), on the proportion of flies that were arrhythmic (Fig4.3B), or the daytime activity of flies (Fig.4.3D). Clothianidin increased night-time activity levels.

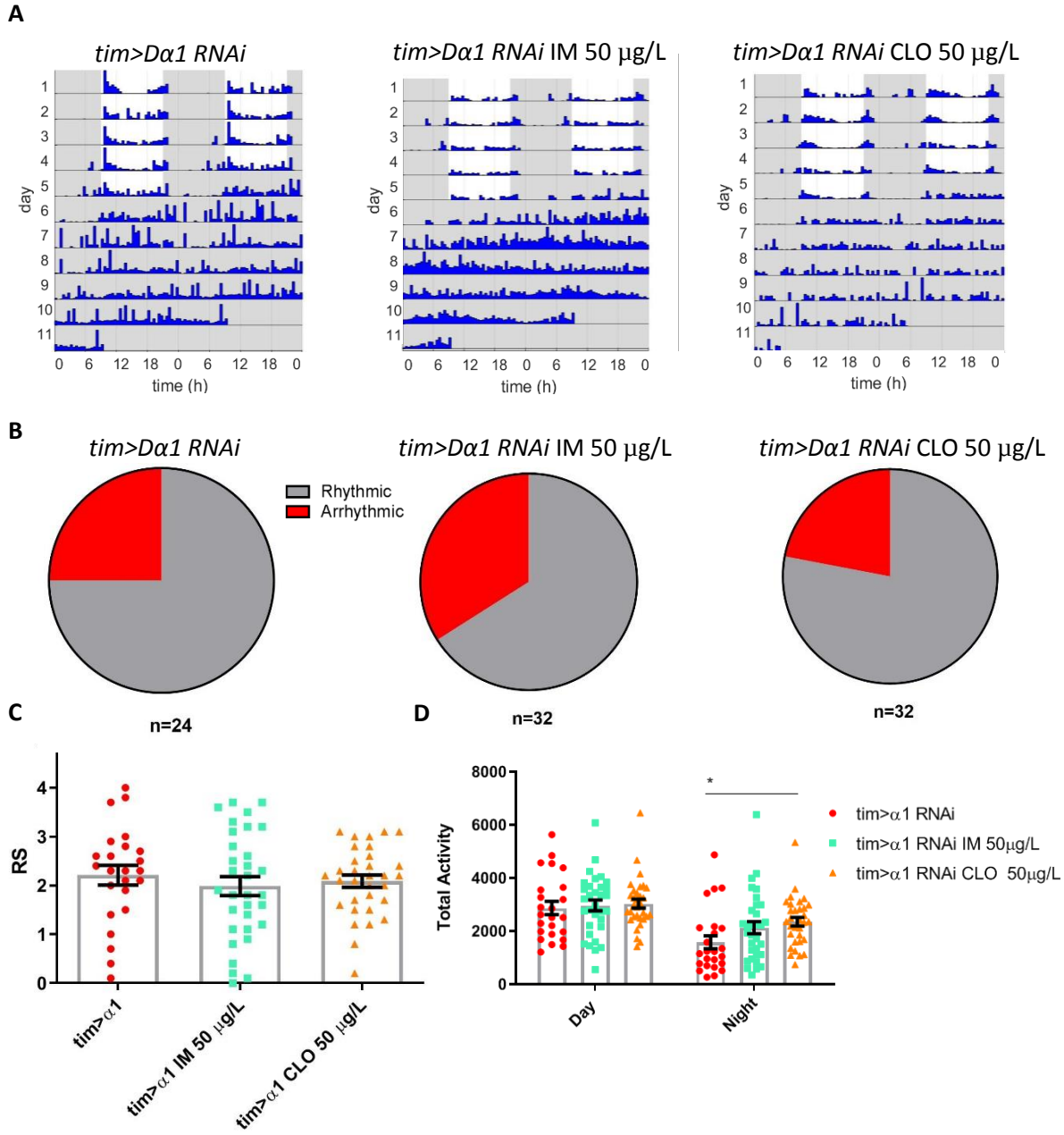

Figure 4.3 Exposure of Dα1 knock down flies to neonicotinoids did not affect rhythmicity

A) Representative actograms for *tim>Dα1 RNAi* on control food or 50µg/L imidacloprid (IM) or clothianidin (CLO). **B)** Proportion of flies that were arrhythmic for *tim>Dα1 RNAi* on control food compared to 50µg/L IM (χ^2_1=2.1, p=0.14) or CLO (χ^2_1=0.4, p=0.5, p=0.53), **C)** Mean behavioural rhythmicity for each genotype ($F_{2,85}$=0.4, p=0.677). **D)** Total daily activity for each treatment during the day ($F_{2,85}$=0.16, p=0.860) and the night ($F_{2,85}$=3.2, p=0.047). Each data point in the histogram represents a single fly, n=19-32 flies per treatment.

However, exposure to neonicotinoids did cause an additive effect on sleep in Dα1 knockdown flies. Exposure to 50µg/L of either imidacloprid or clothianidin caused a visible reduction in daytime sleep (Fig. 4.4A-B). Sleep structure was also significantly affected by neonicotinoid exposure. Knock down flies that were exposed to imidacloprid or clothianidin had significantly more fragmented sleep, with more night-time sleep episodes initiated (Fig. 4.4C) and with the length of sleep episodes being shorter in both the day and night (Fig. 4.4D).

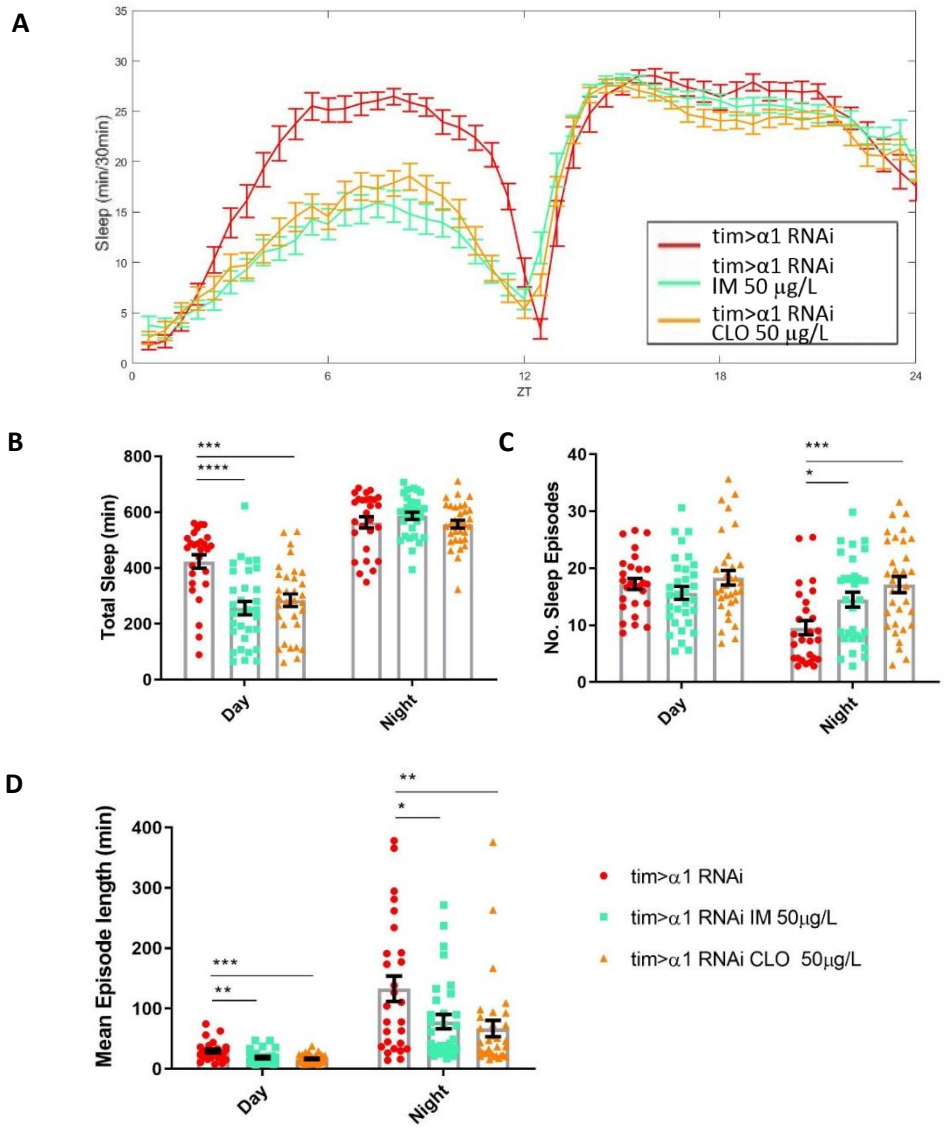

Figure 4.4 Exposure of Dα1 knock down flies to neonicotinoids affected sleep

A) Average sleep achieved by *tim>α1* on control food or exposed to 50 µg/L of imidacloprid (IM) or clothianidin (CLO) over the 24 hour period. **B)** Total amount of sleep achieved by each treatment group during the day ($F_{2,88}$=13.5, p≤0.001) and the night ($F_{2,88}$=1.1, p=0.342). **C)** no. of sleep episodes initiated for each treatment group in the day ($F_{2,88}$=1.4, p=0.254) and night ($F_{2,88}$=7.9, p=0.001) and **D)** mean sleep episode length for each treatment group in the day ($F_{2,88}$=9.8, p≤0.001) and the night ($F_{2,88}$=4.9, p=0.009). For the histograms, each data point represents a single fly, n=29-32 flies for each genotype.

4.2.3 *RNAi* mediated knock down of Dα3 in the clock reduced rhythmicity and disrupted sleep

Knock down of Dα3 in the clock neurons reduced the mean rhythmicity of flies to approximately 1.5, below which flies are characterised as arrhythmic[259] (Fig. 4.5A,C). This was much lower than either of the genotype controls (*tim/+* and *Dα3 RNAi/+*). The knock down flies populations had a higher proportion of arrhythmic flies, with 59% of the population being arrhythmic compared to 0% of the *GAL4* alone control flies (*tim/+*) or 13% for the *UAS* alone control flies (*Dα3 RNAi/+*), (Fig. 4.5B). Both *tim/+* and knockdown flies were more active in day and night time than *Dα3 RNAi/+* flies.

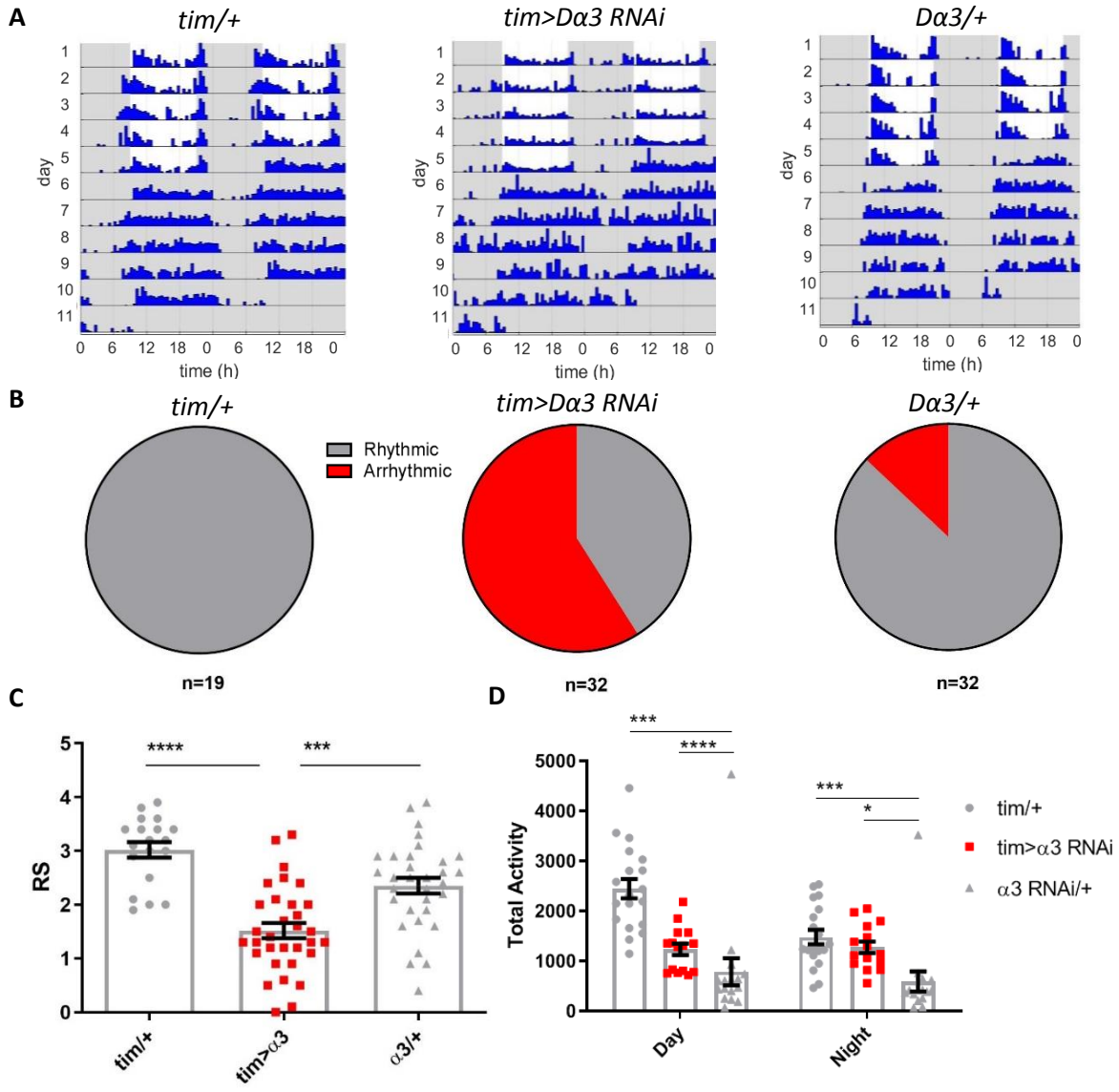

Figure 4.5 Knockdown of Dα3 in the clock reduced rhythmicity

A) Representative actograms for *tim/+, tim>Dα3 RNAi* and *Dα3 RNAi/+* **B)** Proportion of flies that were arrhythmic for *tim>Dα3 RNAi,* compared to *tim/+* (χ^2_1=84.7, p≤0.001)*,* and *Dα3 RNAi/+* (χ^2_1=48.5, p≤0.001). **C)** Mean behavioural rhythmicity for each genotype ($F_{2,80}$=23.8, p≤0.001). **D)** Total daily activity for each genotype during the day ($F_{2,47}$=18.3, p≤0.001) and the night ($F_{2,47}$=8.7, p≤0.001). Each data point in the histogram represents a single fly, n=19-32 flies per treatment.

The knock down of Dα3 in the clock neurons reduced the quantity of sleep achieved by flies, with night-time sleep being reduced compared to *tim/+* flies (Fig. 4.6A-B). Knockdown flies also showed a fragmentation of sleep. They initiated more night-time sleep episodes than either of the genotype controls (Fig. 4.6C) and had shorter sleep episodes than *Dα3/+* (Fig. 4.6D). They also initiated more daytime sleep episodes than *tim/+* (Fig. 4.6C).

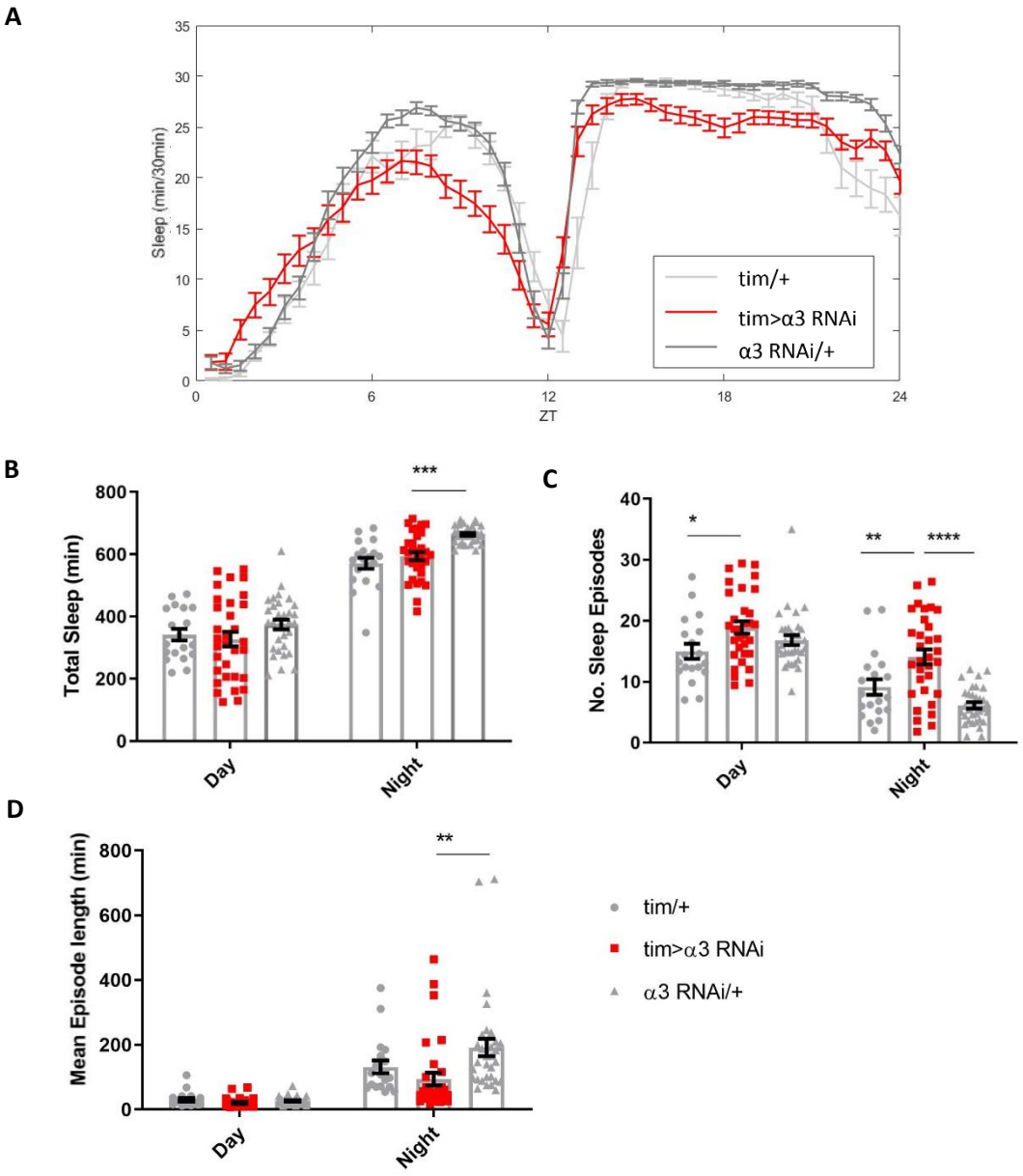

Figure 4.6 Knock down of Dα3 in the disrupted sleep

A) Average sleep achieved by each genotype over the 24 hour period. **B)** Total amount of sleep achieved by each genotype during the day ($F_{2,80}$=1.6, p=0.199) and the night ($F_{2,80}$=16.6, p≤0.001). **C)** no. of sleep episodes initiated for each genotype in the day ($F_{2,80}$=3.5), p=0.035) and night ($F_{2,80}$=17.6, p≤0.001) and **D)** mean sleep episode length in day ($F_{2,80}$=2.3, p=0.105) and night ($F_{2,80}$=5.0, p=0.009). For the histograms, each data point represents a single fly, n=19-32 flies for each genotype.

4.2.4 Neonicotinoid exposure in Dα3 knock down flies caused further disruption to sleep

The exposure of Dα3 knock down flies to 50 µg/L of imidacloprid or clothianidin did not further reduce behavioural rhythmicity (Fig.4.7A,C). Exposure to imidacloprid failed to increase the proportion of the population that were arrhythmic, with 50% of knock down flies being arrhythmic, compared to 59%, whereas clothianidin decreased arrhythmicity, to 39% (Fig.4.7B). Both imidacloprid and clothianidin decreased activity in the day and night (Fig.4.7D).

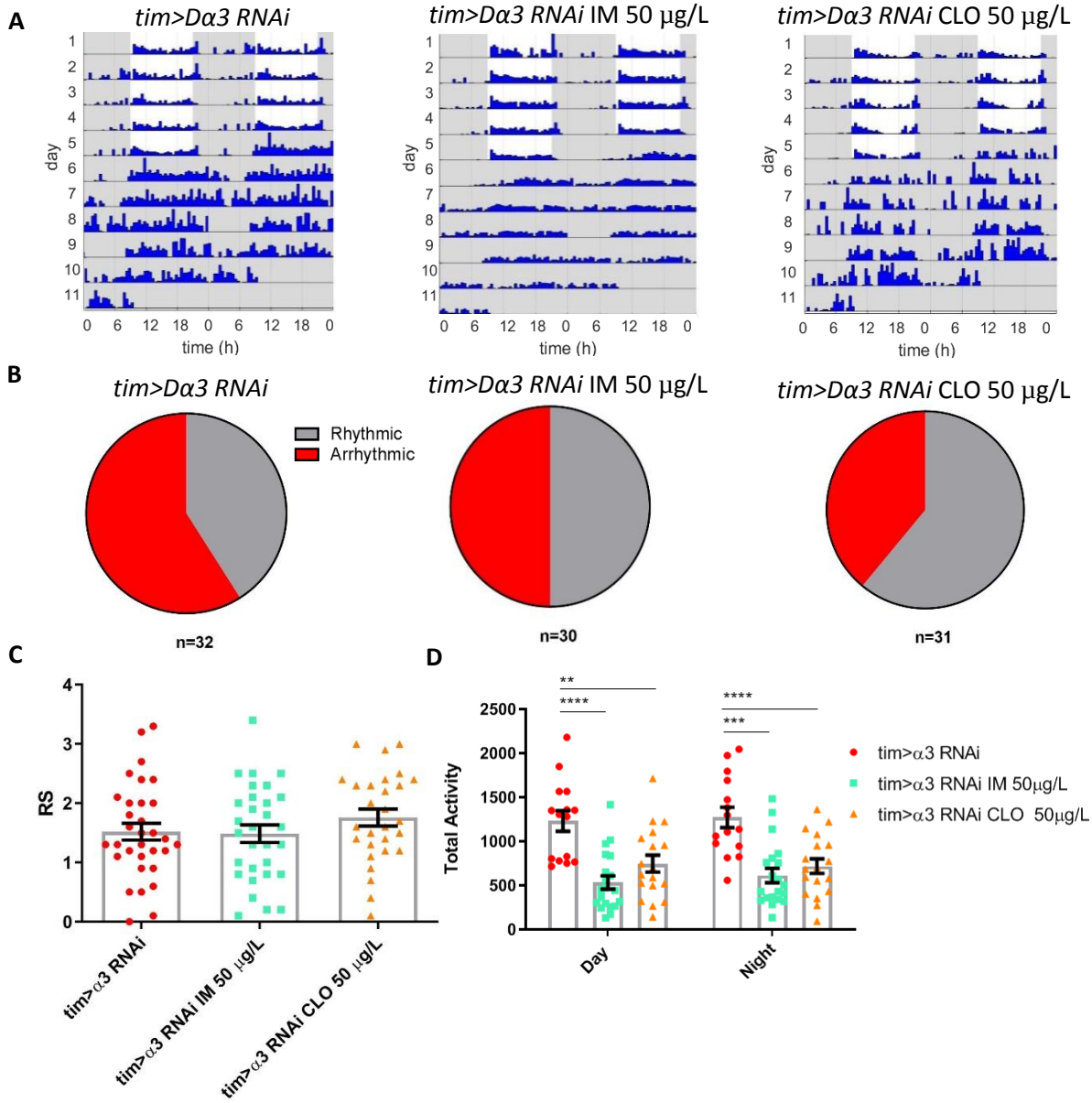

Figure 4.7 Exposure of Dα3 knockdown flies to neonicotinoids did not affect rhythmicity

A) Representative actograms for *tim>Dα3 RNAi*, *tim> Dα3 RNAi* 50 µg/L imidacloprid (IM) and *tim> Dα3 RNAi* 50 µg/L clothianidin (CLO). **B)** Proportion of flies that were arrhythmic for *tim>Dα3 RNAi* flies on control food compared to 50 µg/L IM (χ^2_1=1.9, p=0.17) or CLO (χ^2_1=8.9, p=0.003). **C)** Mean behavioural rhythmicity for each treatment group ($F_{2,87}$=1.0, p=0.370). **D)** Total daily activity for each treatment during the day ($F_{2,50}$=13.6, p≤0.001) and the night ($F_{2,50}$=13.9, p≤0.001). Each data point represents a single fly, n=19-32 flies per treatment.

Additional exposure to imidacloprid had no effect on the sleep behaviour of Dα3 knock down flies (Fig. 4.8). However, exposure to clothianidin caused additional changes to many aspects of sleep behaviour. Clothianidin exposure resulted in increased day time sleep (Fig. 4.8A-B), fewer night-time sleep episodes (Fig. 4.8C) and longer daytime sleep episodes than in unexposed Dα3 knockdown flies (Fig. 4.8D).

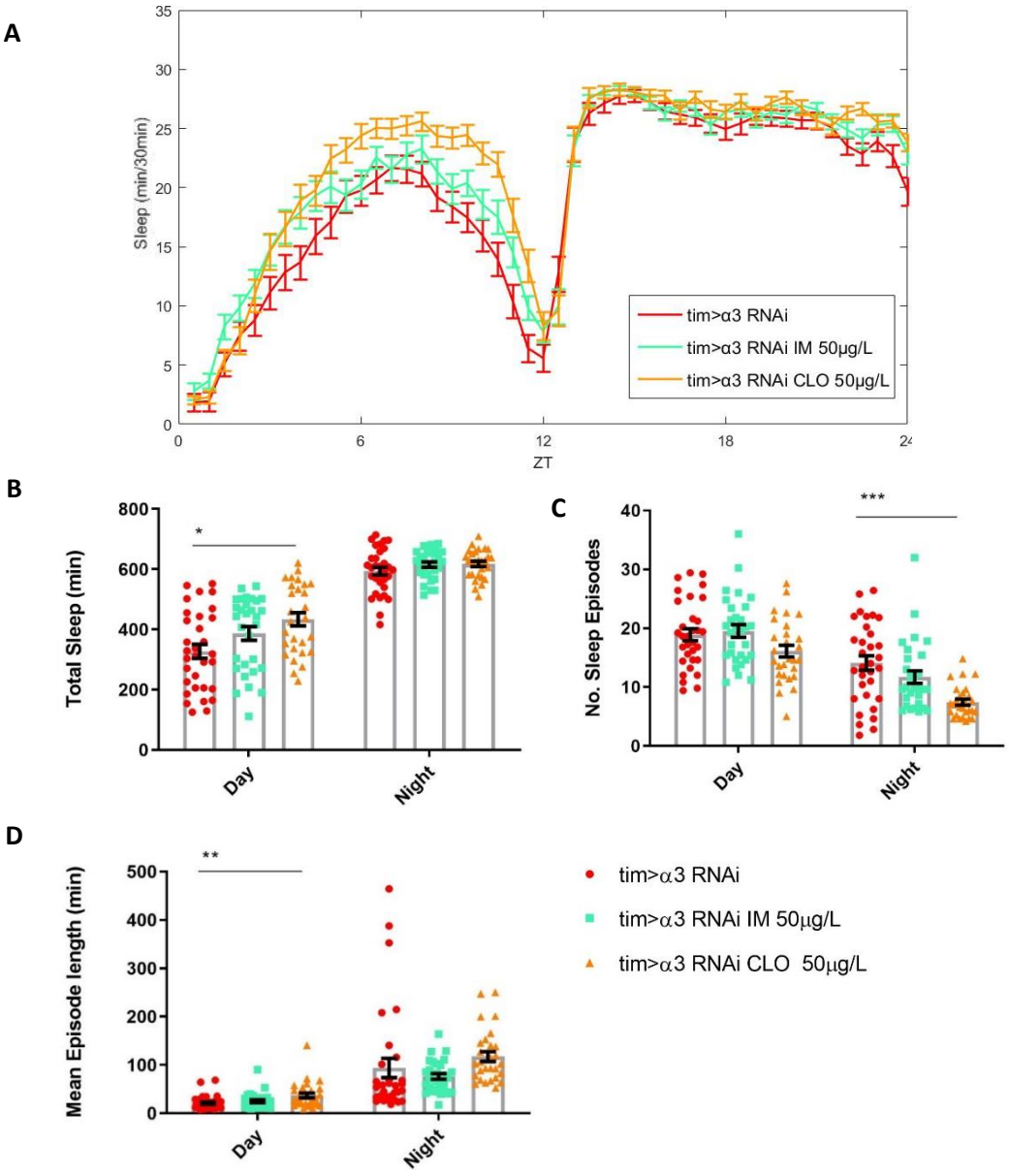

Figure 4.8 Exposure of Dα3 knock down flies to neonicotinoids affected sleep

A) Average sleep achieved by *tim> Dα3* on control food or exposed to 50µg/L of imidacloprid (IM) or clothianidin (CLO) over the 24 hour period, **B)** Total amount of sleep achieved by each treatment group during the day ($F_{2,87}$=5.5, p=0.006) and the night ($F_{2,87}$=1.5, p=0.221), **C)** number of sleep episodes initiated for each treatment group in the day ($F_{2,87}$=3.0), p=0.053) and night ($F_{2,87}$=11.0, p≤0.001) and **D)** mean sleep episode length for each treatment group in the day ($F_{2,87}$=5.1, p=0.008) and the night ($F_{2,87}$=2.1, p=0.124). For the histograms, each data point represents a single fly, n=29-32 flies for each genotype.

4.2.5 *RNAi* mediated knock down of Dβ2 in the clock reduced rhythmicity and disrupted sleep

The knock down of Dβ2 in the clock neurons caused a large decrease in rhythmicity in constant conditions (Fig. 4.9A,C). The knock down flies had a much lower rhythmicity strength than either of the control genotypes, with a mean rhythmicity of approximately 1, denoting arrhythmicity[259] (Fig. 4.9C). A much larger proportion, 71%, of these flies were arrhythmic, compared to 34% of the *UAS* alone control flies (*Dβ2 RNAi/+*) and 0% of the *GAL4* control flies (*tim/+*) (Fig. 4B). Knockdown flies also had increased night-time activity levels compared to both control genotypes.

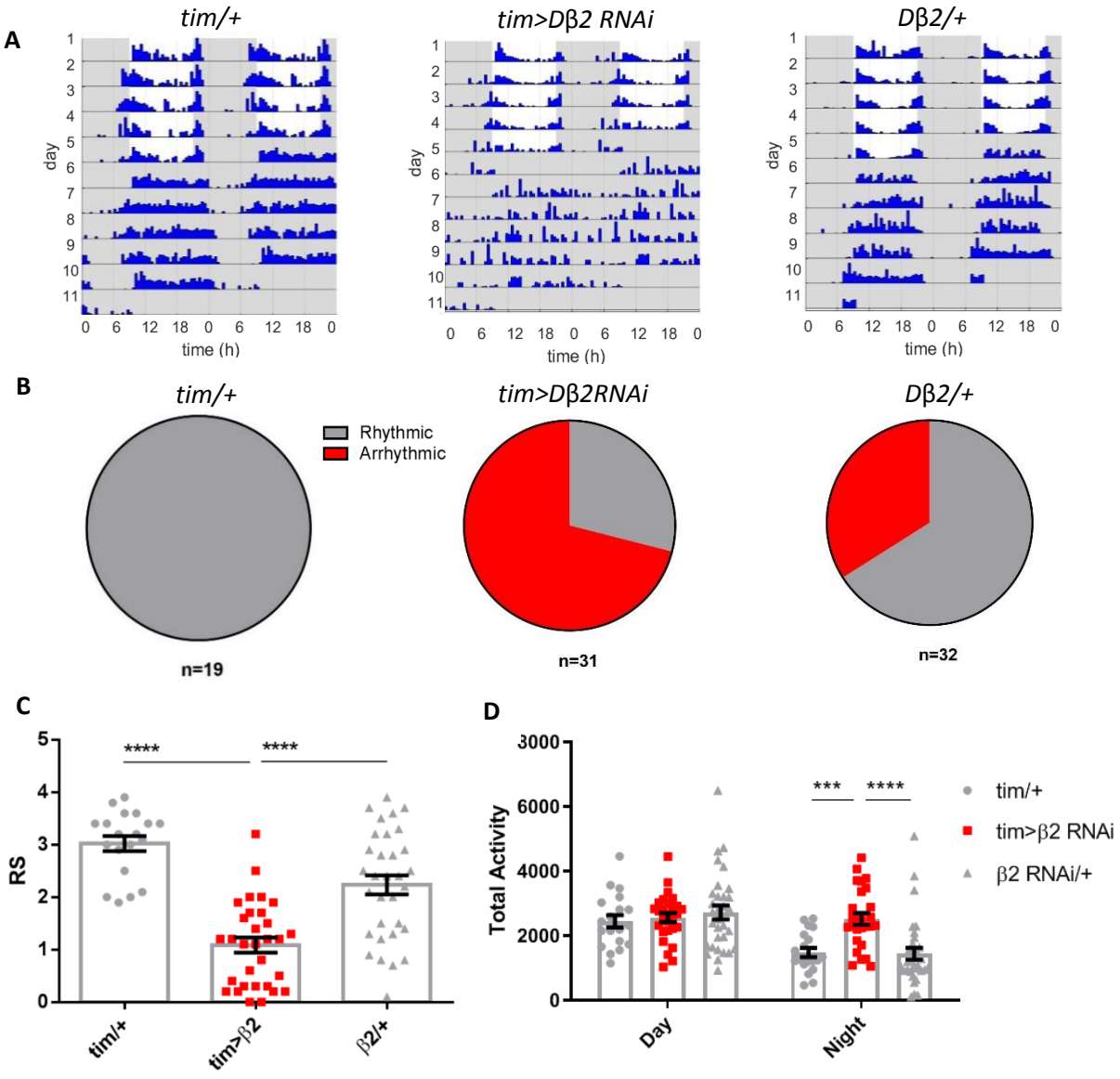

Figure 4.9 Knock down of Dβ2 in the clock reduced rhythmicity

A) Representative actograms for *tim/+*, *tim>Dβ2 RNAi* and *Dβ2 RNAi/+*. **B)** Proportion of flies that were arrhythmic for *tim>Dβ2 RNAi*, compared to *tim/+* (χ^2_1=109.1, p≤0.001), and *Dβ2 RNAi/+* (χ^2_1=26.3, p≤0.001). **C)** Mean behavioural rhythmicity for each genotype ($F_{2,79}$=31.5, p≤0.001), D**)** Total daily activity for each genotype during the day ($F_{2,75}$=0.5, p=0.620) and the night ($F_{2,75}$=11.8, p≤0.001). Each data point in the histogram represents a single fly, n=19-32 flies per treatment.

The knock down of Dβ2 also caused significant changes to sleep behaviour. These flies achieved less sleep than control flies, sleeping less in the day than both control genotypes and having less night-time sleep than the *tim/+* flies (Fig. 4.10A-B). Knock down flies also showed sleep fragmentation, initiating more night-time sleep episodes than either control genotype (Fig. 4.10C) and having shorter sleep episodes in both daytime and night-time (Fig. 4.10D).

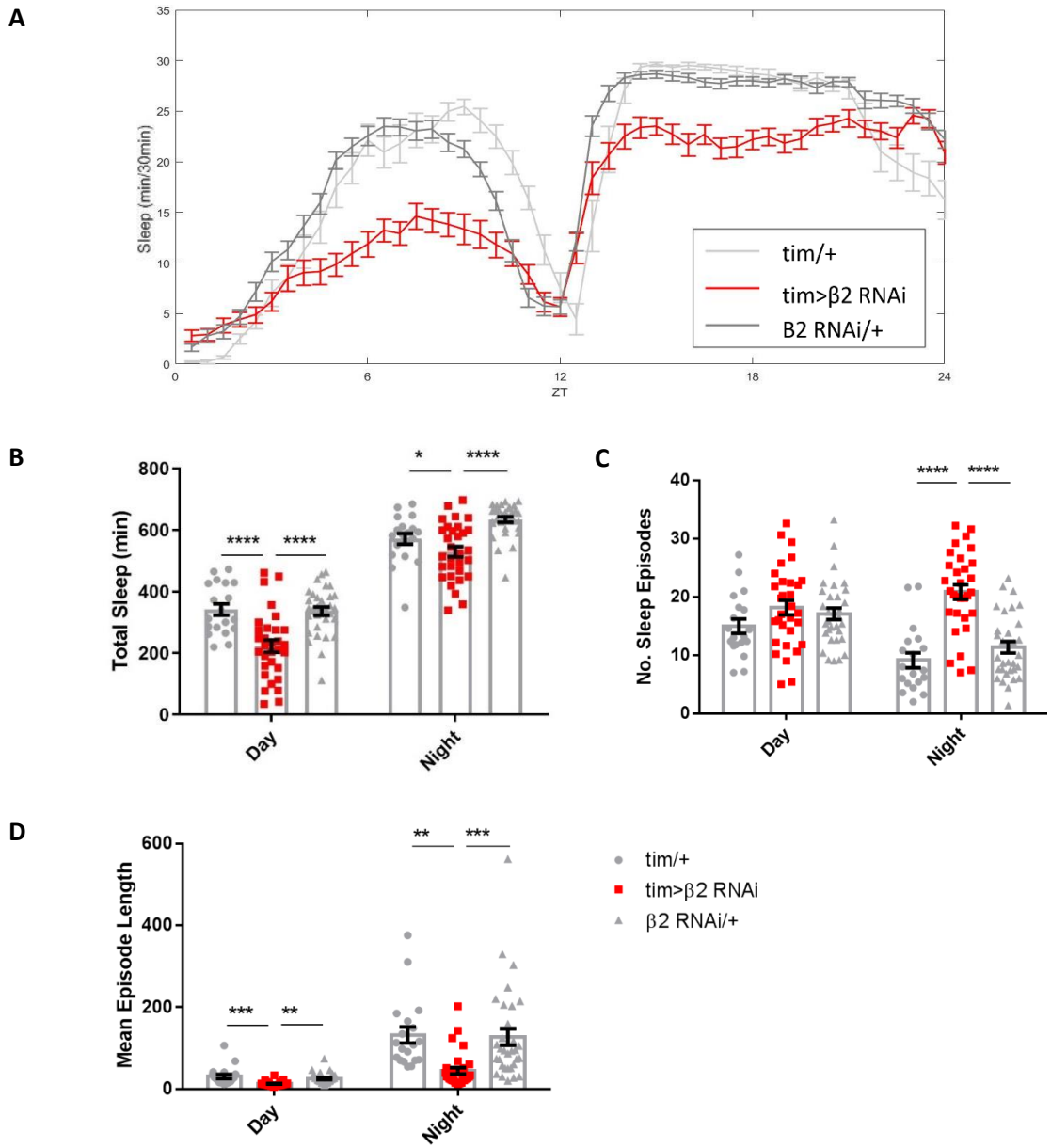

Figure 4.10 Knock down of Dβ2 in the clock disrupted sleep

A) Average sleep achieved by each genotype over the 24 hour period. **B)** Total amount of sleep achieved by each genotype during the day ($F_{2,79}$=15.3, p≤0.001) and the night ($F_{2,79}$=15.2, p≤0.001). **C)** number of sleep episodes initiated for each genotype in the day ($F_{2,79}$=1.6), p=0.211 and night ($F_{2,79}$=28.2, p≥0.001) and **D)** mean sleep episode length in day ($F_{2,79}$=11.2, p≤0.001) and night ($F_{2,79}$=9.4, p≤0.001), for the histograms, each data point represents a single fly, n=19-32 flies for each genotype.

4.2.6 Neonicotinoid exposure in Dβ2 knock down flies caused further disruption to sleep

Flies with Dβ2 knockdown in the clock neurons who were then exposed to 50 µg/L of either imidacloprid or clothianidin showed no further change in mean rhythmicity (Fig.4.11A,C). Exposure to imidacloprid or clothianidin didn't cause a significant increase in the proportion of the population that were arrhythmic, 25%, compared to 38% and 31% respectively (Fig.4.11B). Neither neonicotinoid affected daytime activity, however clothianidin reduced night activity (Fig.4.11D).

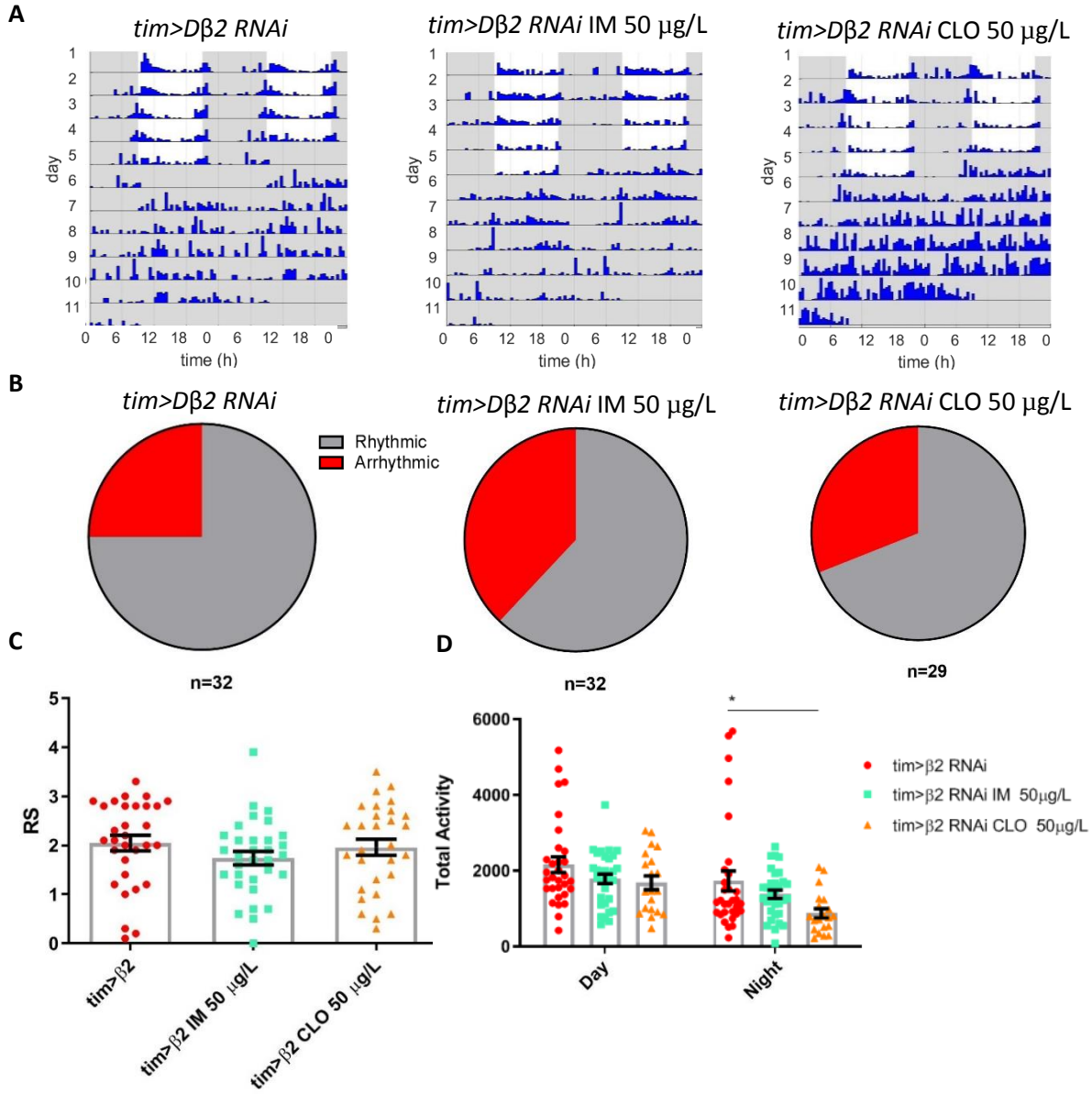

Figure 4.11 Exposure of Dβ2 knock down flies to neonicotinoids did not affect rhythmicity

A) Representative actograms for *tim>Dβ2 RNAi* on control food or 50 µg/L of imidacloprid (IM) or clothianidin (CLO). **B)** Proportion of flies that were arrhythmic for *tim>Dβ2 RNAi* on control food compared to 50 µg/L IM (χ^2_1=3.4, p=0.066) or CLO (χ^2_1=0.9, p=0.340) **C**) Mean behavioural rhythmicity for each treatment group ($F_{2,89}$=1.1, p=0.336). **D)** Total daily activity for each genotype during the day ($F_{2,80}$=2.1, p=0.130) and the night ($F_{2,80}$=4.3, p=0.018). Each data point in the histogram represents a single fly, n=19-32 flies per treatment.

Exposure of Dβ2 knock down flies to 50 µg/L of clothianidin had no effect on sleep behaviour (Fig. 4.12). Exposure to 50 µg/L of imidacloprid had no effect on night-time sleep (Fig. 4.12A-B) and did not affect the number of sleep episodes initiated (Fig. 4.12C). However, imidacloprid exposure did cause a reduction in total daytime sleep (Fig. 4.12A-B) and a small reduction in the length of night-time sleep episodes in knock down flies (Fig. 4.12D).

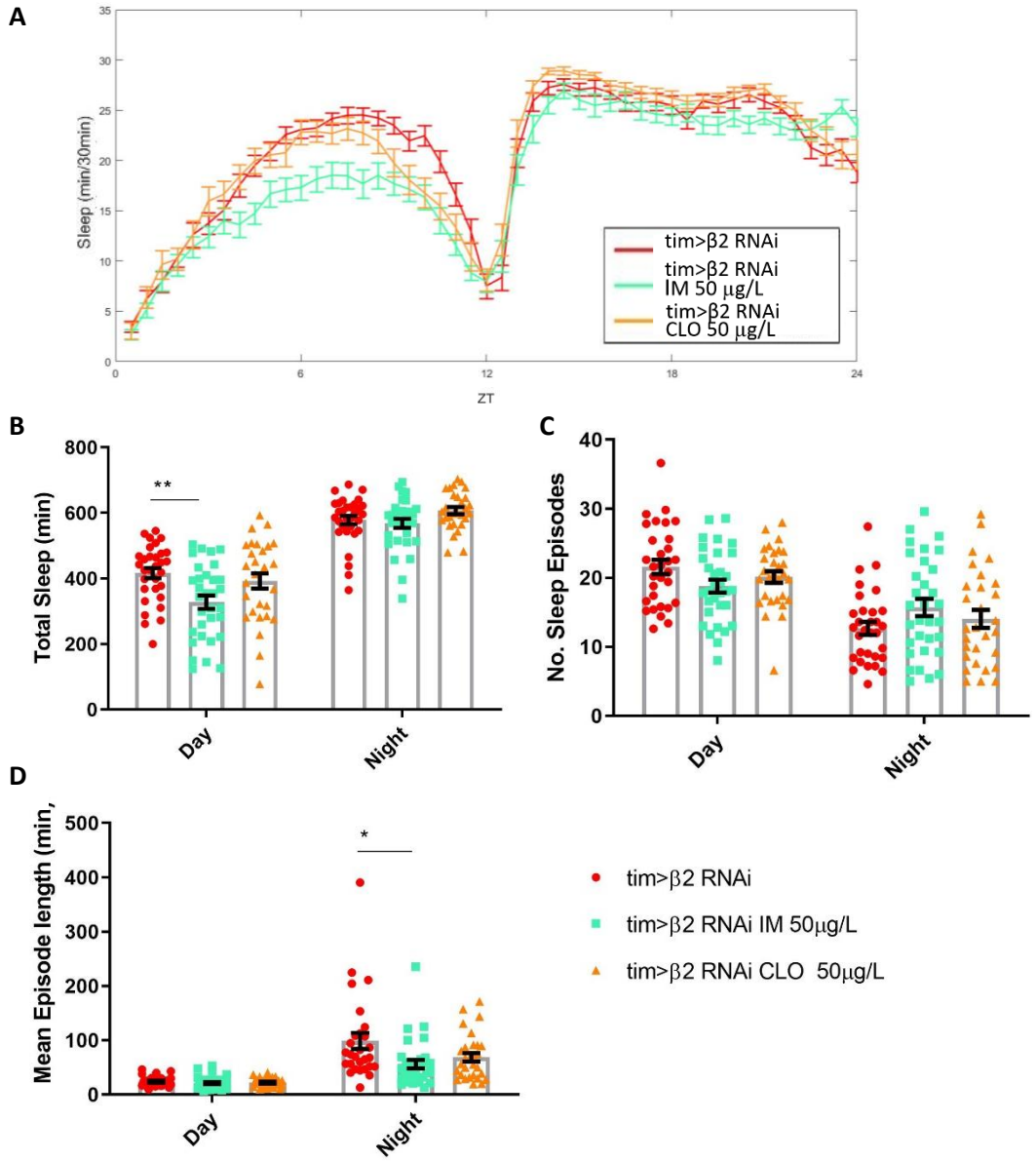

Figure 4.12 Exposure of Dβ2 knock down flies to neonicotinoids did affect sleep

A) Average sleep achieved by *tim>Dβ2 RNAi* on control food or exposed to 50 µg/L of imidacloprid (IM) or clothianidin (CLO) over the 24 hour period. **B)** Total amount of sleep achieved by each treatment group during the day ($F_{2,89}$=5.6, p=0.005) and the night ($F_{2,89}$=2.4, p=0.096). **C)** number of sleep episodes initiated for each treatment group in the day ($F_{2,89}$=2.3), p=0.108 and night ($F_{2,89}$=1.7, p=0.180) and **D)** mean sleep episode length for each treatment group in the day ($F_{2,89}$=0.5, p=0.581) and the night ($F_{2,89}$=4.4, p=0.015). For the histograms, each data point represents a single fly, n=29-32 flies for each genotype.

4.3 Neonicotinoid exposure reduced the circadian plasticity and PDF cycling in the s-LNv dorsal terminals

4.3.1 Neonicotinoid exposure reduced circadian remodelling

Exposure to 50 µg/L of either imidacloprid or clothianidin significantly reduced the circadian plasticity of the s-LNv dorsal terminals (Fig. 4.13A). Whereas control flies showed greater axonal branching in the day than at night, neonicotinoid exposed flies did not (Fig. 4.13A). Their terminals had a highly branched structure during both the day and the night, resembling that seen in control flies in daytime (Fig. 4.14).

4.3.2 Neonicotinoid exposure reduced PDF cycling

Neonicotinoid exposure also prevented the daily cycling in PDF accumulation in the s-LNv dorsal terminals (Fig. 4.13B). In control flies, PDF accumulation was approximately twice as high in the day than it was at night (Fig. 4.13B). However, in flies exposed to neonicotinoids there was no significant difference between day and night-time levels (Fig. 4.13B, 4.14).

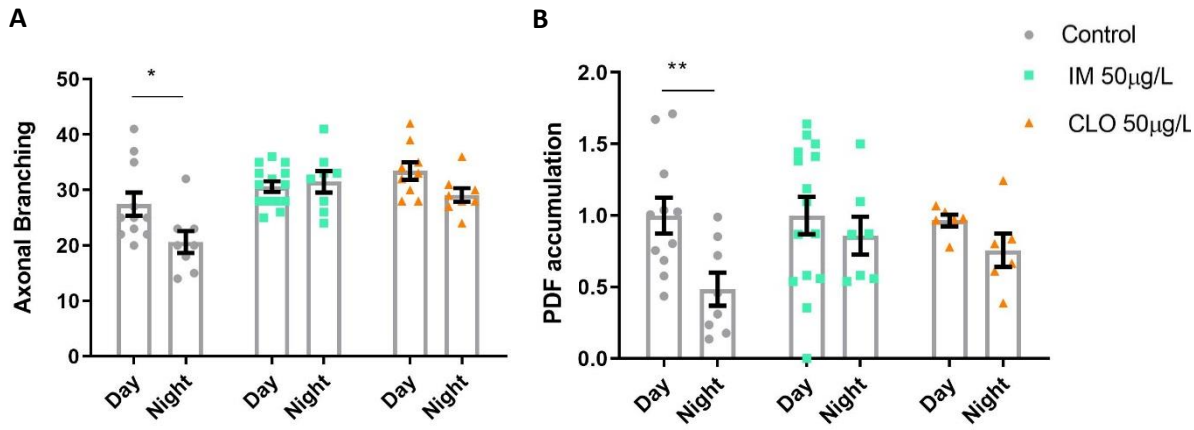

Figure 4.13 Neonicotinoids reduced circadian plasticity and PDF cycling in the s-LNv dorsal terminals

A) Day night differences in s-LNv dorsal terminals branching complexity for control flies (t_{17}=2.3, p=0.036), flies exposed to 50 µg/L of imidacloprid (IM) (t_{14}=2.1, p=0.055) and flies exposed to 50µg/L of clothianidin (CLO) (t_{15}=2.1, p=0.052). **B)** Day night differences in PDF accumulation in the s-LNv dorsal terminals for control flies ($t_{(17)}$=2.9, p=0.010), flies exposed to 50 µg/L of imidacloprid (IM) (t_{13}=1.0, p=0.332) and flies exposed to 50µg/L of clothianidin (CLO) (t_{14}=2.1, p=0.054). Each data point represents a single brain, n=6-15 brains per treatment.

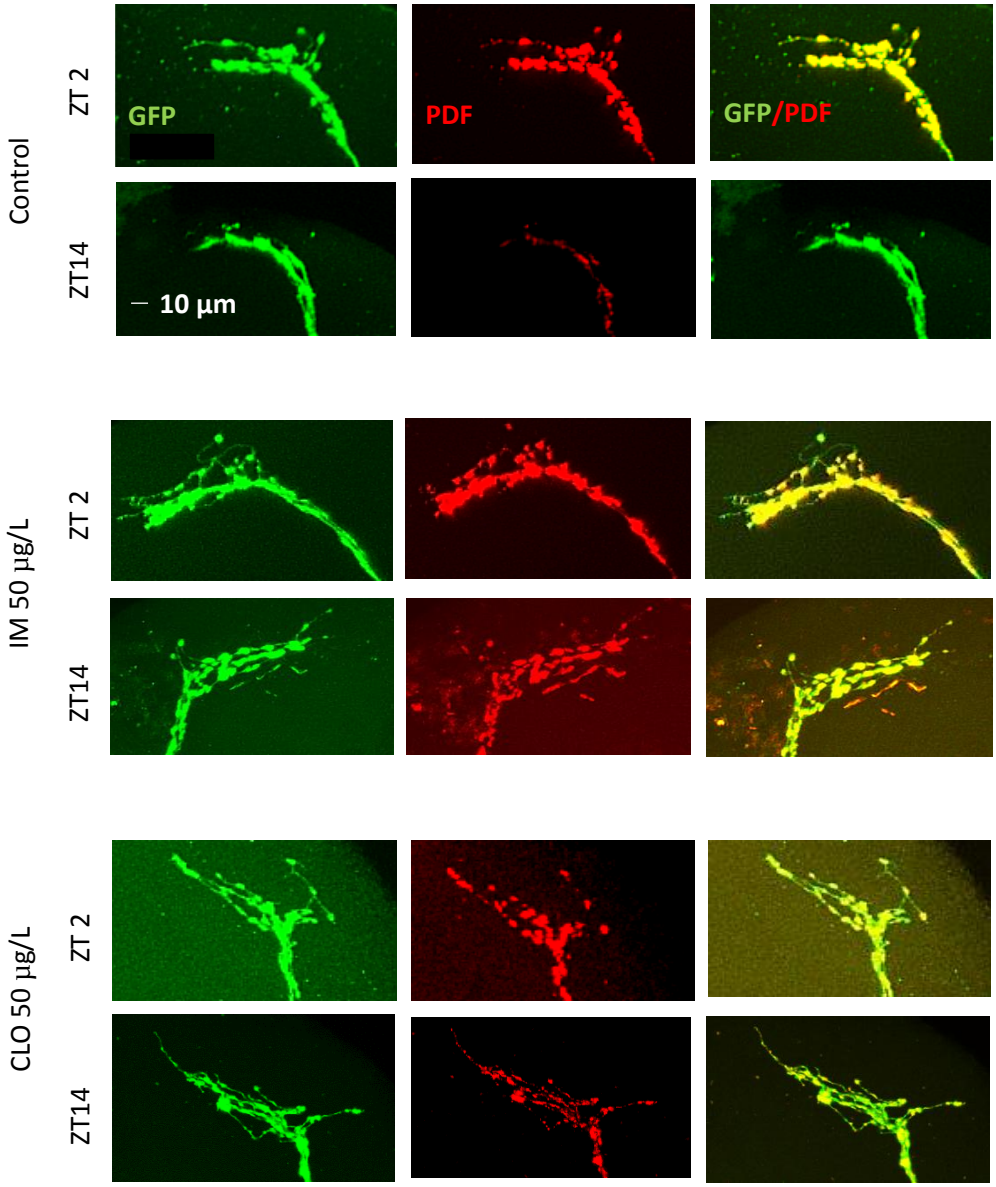

Figure 4.14 Neonicotinoids reduced circadian plasticity and PDF cycling in the s-LNv dorsal terminals

Representative examples of confocal image of the s-LNv dorsal terminals of a control fly, a fly exposed to 50 µg/L of imidacloprid (IM) and a fly exposed to 50 µg/L of clothianidin, showing the axonal structure in GFP in green, the PDF in red, and then GFP and PDF merged, for both the morning (ZT 2) and night (ZT 14).

4.4 *RNAi* mediated knockdown of Dα1 or Dβ2 reduced the circadian plasticity and PDF cycling in the s-LNv dorsal terminals

4.4.1 *RNAi* mediated knock down of Dα1 or Dβ2 reduced circadian remodelling

The knock down of either Dα1 or Dβ2 in the LNvs prevented the daily remodelling of the s-LNv dorsal terminals (Fig. 4.15A). The terminals of flies with a knock down of either of these subunits showed a low level of branching during both the day and night and which was comparable to that seen in control flies at night (Fig. 4.16).

4.4.2 *RNAi* mediated knock down of Dα1 or Dβ2 reduced PDF cycling

The knock down of either Dα1 or Dβ2 in the LNvs also reduced the daily changes in PDF accumulation in the s-LNvs (Fig. 4.15B). Unlike in control flies, there was no significant difference in PDF accumulation during the day compared to the night (Fig. 4.15B, 4.16).

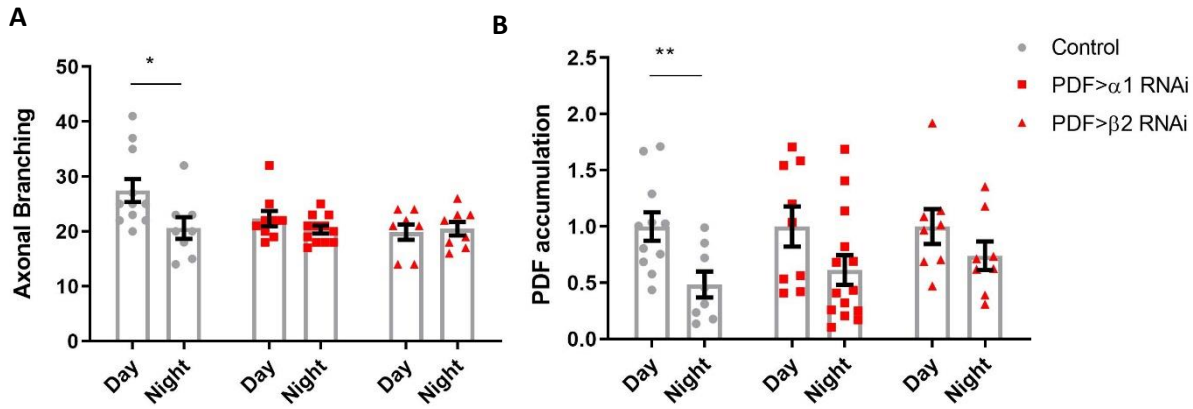

Figure 4.15 Knockdown of Dα1 or Dβ2 in LNvs reduced circadian plasticity and PDF cycling in the s-LNv dorsal terminals

A) Day/night differences in s-LNv dorsal terminals branching complexity for control flies (t_{17}=2.3, p=0.036), flies with knock down of Dα1 in the LNvs (t_{19}=1.4, p=0.183) and flies with knock down of Dβ2 in the LNvs (t_{13}=-0.7, p=0.515). **B)** Day night differences in PDF accumulation in the s-LNv dorsal terminals for control flies (t_{17}=2.9, p=0.010), flies with knockdown of Dα1 in the LNvs (t_{19}=1.8, p=0.089) and flies with knock down of Dβ2 in the LNvs (t_{14}=1.3, p=0.218). Each data point represents a single brain, n=6-15 brains per treatment.

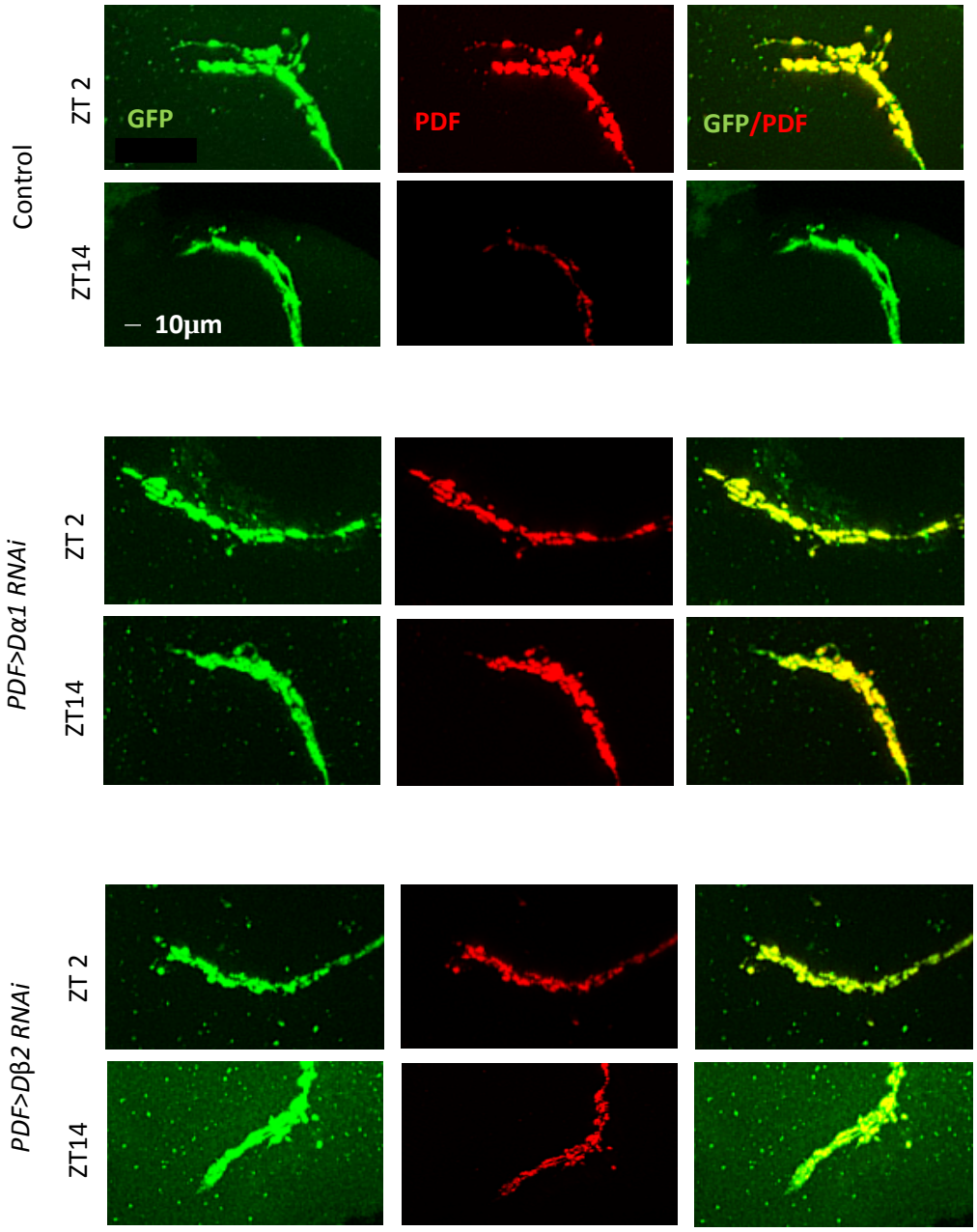

Figure 4.16 Knockdown of Dα1 or Dβ2 in LNvs reduced circadian plasticity and PDF cycling in the s-LNv dorsal terminals

Representative examples of confocal image of the s-LNv dorsal terminals of a control fly, a fly with Dα1 knocked down in the LNvs and a fly with Dβ2 knocked down in the LNvs, showing the axonal structure in GFP in green, the PDF in red, and then GFP and PDF merged, for both the morning (ZT 2) and night (ZT 14).

4.5 Discussion

This discussion focuses on the effects of Dα1, Dα3 and Dβ2 knock down on rhythmicity and sleep in *Drosophila* and the effects of these knock downs and neonicotinoid exposure on the circadian plasticity of the s-LNvs. The broader consequences of these finding and their possible field relevance will then be discussed in Chapter 6.

4.5.1 *RNAi* mediated knock down of Dα1, Dα3 or Dβ2 in the clock altered activity levels

For Dα1 and Dα3, the effect of knockdowns of activity appeared to be dependent on the activity of the two parent lines, with activity levels for the knockdown falling between these. However, for Dβ2 knockdowns there was a clear effect, with knockdowns exhibiting more night-time activity than either control. Due to the varied effects of neonicotinoids on activity in wildtype flies, it is hard to compare these with the activity of knock down flies. Exposure of knock down flies to imidacloprid or clothianidin also had varied effects on activity. In Dα3 knock downs, both neonicotinoids caused a reduction in activity levels in both daytime and night-time. In Dα1 and Dβ2 knockdowns, imidacloprid had no effect, whereas clothianidin caused an increase in night-time activity in Dα1 knockdowns and a decrease in night-time activity in Dβ2 knockdowns. Clearly, as discussed in 3.6.2 for wildtype flies, neonicotinoid exposure can cause a range of effects on activity in knock down flies.

4.5.2 *RNAi* mediated knock down of Dα1, Dα3 or Dβ2 in the clock disrupted circadian rhythmicity

The knock down of Dα1, Dα3 or Dβ2 nAChR subunits in the clock neurons reduced behavioural rhythmicity under constant conditions. For Dα3 or Dβ2, rhythmicity in knockdown flies was significantly lower than both genotype controls. For Dα1, the *UAS* only control also showed some reduction in rhythmicity, suggesting that it may be leaky. However, the reduction in rhythmicity for both genotypes in constant conditions suggest that Dα1 is important for rhythmicity and the effect that Dα1 knockdown had on the s-LNv dorsal terminals suggests that it occurs in the central clock. The results for Dα3 and Dβ2 also suggest that they occur in the clock neurons and are involved in the function of the central clock.

The s-LNvs, the clock neurons that maintain the clock under constant conditions, are nicotinic[90,151]. Previous work has shown that electrical silencing of the LNvs in flies causes behavioural arrhythmicity in constant conditions[166]. This may be because electrical silencing appears to prevent the circadian cycling of PDF accumulation in the s-LNv dorsal terminals[151], which may affect communication with downstream clock neurons, including the DN1s. The DN1's connect the s-LNvs with neurons in the *pars intercerebralis,* which are important for locomotion and arousal[159]. The day-

night difference in electrical activity in the LNvs is also important for maintaining the molecular clock under constant conditions, with the molecular clock rapidly running down in flies with electrically silenced LNvs[167]. The knock down of the Dα1, Dα3 or Dβ2 nAChR subunits may cause electrical silencing of the nicotinic LNvs, changing or preventing the formation of nAChRs and reducing excitation of these cells by ACh from the light sensing organs during the entrainment period. This could explain the loss of behavioural rhythmicity observed in knock down flies under constant conditions. As the s-LNvs are the main cells that drive rhythmicity under constant conditions, they seem like good candidates for explaining this behavioural change. Other than the LNvs, only a small subset of clock neurons, the LNds, have been shown to use ACh signalling, and this appears to be for communication with the LNvs[231].

The effect on rhythmicity observed for these knock down flies was very similar to the effect observed for flies exposed to neonicotinoids in Chapter 3. Furthermore, the addition of neonicotinoids to the Dα1, Dα3 and Dβ2 nAChR subunit knock downs did not cause any further loss of rhythmicity. This suggests that the Dα1, Dα3 and Dβ2 subunits occur in the receptor or receptors which are susceptible to neonicotinoids in the clock neurons. Previous work in *Drosophila* has shown that Dα1 and Dβ2 are involved in neonicotinoid susceptibility[84]. Whilst Dα3 has not previously been shown to have sensitivity to neonicotinoids in *Drosophila*, Nα1 and Nα3 have been shown to be involved in neonicotinoid susceptibility in the brown leafhopper (*N. Lugens*)[81,290].

The lack of additive effect under neonicotinoid exposure may also suggest that Dα1, Dα3 and Dβ2 all occur in the same receptor subtype/s, and that this might be the only neonicotinoid susceptible subtype/s present in the central clock. A functional pentameric channel involving these three has not yet to be identified, but this may be because functional insect nAChRs have proven very difficult to express, limiting out knowledge in this area[72]. It has previously been postulated that these subunits could co-occur in receptors; co-expression and co-precipitation data suggests three likely compositions: one involving at least Dβ1 and Dβ2 and an α subunit, one containing at least Dα1, Dα2 and Dβ2 and one with at least Dβ1 and Dα3[74]. It is possible that one of these could contain all three of the subunits tested. The lack of further loss of rhythmicity in neonicotinoid exposed knock down flies also suggests that neonicotinoids may be disrupting rhythmicity by interacting directly with the clock. If the neonicotinoids were also acting elsewhere in the brain or body then neonicotinoid exposure would likely have an additive effect on rhythmicity, which was not observed.

No nAChR subunits have been shown to play a specific role in circadian rhythmicity in insects before. The effects of *rye* have previously been tested and no circadian effect was shown, which may

suggest that *rye* is not Dα3[86,291] but rather one of the other nAChRs with which it has homology such as Dα4.

4.5.3 *RNAi* mediated knock down of Dα1, Dα3 or Dβ2 in the clock disrupted sleep

The knock down of either Dα1, Dα3 or Dβ2 caused a disruption to sleep behaviour resembling that seen in neonicotinoid exposed flies in Chapter 3. Knock down flies experienced a fragmentation and loss of sleep. This suggests that the subunits occur in a receptor or receptors in the clock neurons which are involved in normal sleep behaviour. The l-LNvs are clock neurons and key arousal neurons and are involved in the circadian timing of sleep[153,181]. These neurons are nicotinic, relying on ACh signalling to receive excitatory information from the visual system that contributes to the electrical state of these neurons and thus their role in the sleep wake circuitry[91,153]. The l-LNvs show robust day/night differences in their electrical state which appear to be integral to their role as arousal neurons[162]. Previous work has shown that disrupting this day-night difference through hyperexcitation of the l-LNvs caused them to become less responsive to light input and caused shorter sleep episodes and loss of sleep[153]. Possibly the knock down of the nAChR subunits Dα1, Dα3 or Dβ2 is preventing excitation of the l-LNvs, causing electrical silencing of these neurons and resulting in a loss of day/night difference in their electrical state. This could explain the fragmentation and loss of sleep observed in knock down flies. Disruption to day-night differences in the electrical state of the LNvs, whether through hyperexcitation or electrical silencing both appear to disrupt the circadian timing of arousal and activity[165,166], which could result in disrupted sleep maintenance such as that was observed for knock down flies in this chapter. It appears that the relative electrical state of the l-LNvs is more important for the occurrence and timing of arousal and activity than the absolute electrical state of the cells. For example, flies with electrically silenced l-LNvs are arrhythmic but they are not inactive[151].

Another group of clock neurons involved in the sleep wake circuitry are the DN1s. There are a subset of sleep promoting DN1s which extend to the Ellipsoid Body (EB)-R2 neurons and are involved in the sleep homeostat[192], and others that project back to the LNvs, inhibiting their activity to allow sleep during the night[193]. Although the DN1s do not appear to be cholinergic[292] and are thus unlikely to be directly affected by the knock down of nAChR subunits, they are down stream of the LNvs. PDF signalling from the LNvs activates the DN1s, with PDF causing depolarisation and increased firing rate[289]. As PDF cycling is another circadian output which appears to be reliant on the day-night differences in the electrical state of the LNvs[151,165], it is possible that PDF signalling in the knock down flies is disrupted, reducing activation of these sleep promoting DN1s and contributing to the reduced and disrupted sleep observed in knock down flies.

The addition of neonicotinoids to these knock down flies had varying effects. The addition of either imidacloprid or clothianidin caused further fragmentation and loss of sleep in Dα1 knock down flies. The addition of clothianidin had no impact on the sleep of Dβ2 knock downs and imidacloprid had little effect, causing a decrease in daytime sleep and a slight decrease in episode length at night. For Dα3 knock downs, exposure to imidacloprid had no further effect on sleep behaviour, however clothianidin caused an increase in daytime sleep and an increase in the length of daytime sleep episodes.

This suggests that there are multiple subtypes of nAChR that occur in the clock neurons and which are involved in sleep behaviour. These subtypes appear to include one of more which are susceptible to neonicotinoids but that do not involve Dα1, resulting in the further effects on sleep behaviour that neonicotinoid exposure had on Dα1 knock down flies. In *N. lugens* Nα1 is thought to occur in only one of two potential neonicotinoid susceptible nAChRs which have been identified[293].

Dα3 appears to occur in all sleep related nAChR subtypes that are susceptible to imidacloprid in the clock neurons, as imidacloprid exposure caused no further sleep effects in these knock down flies. Similarly, Dβ2 appears to occur in all clothianidin susceptible sleep related nAChR subtypes, as clothianidin exposure had no effect on sleep in these flies. There also appears to be a subtype of receptor complex which does not include Dβ2 and is susceptible to imidacloprid and a subtype that does not contain Dα3 and is involved in clothianidin susceptibility. Previous work on Kenyon cells in bees identified some cells that were susceptible to imidacloprid, some to clothianidin, some to neither and some to both, showing that this sort of specificity does occur[75].

In most cases where neonicotinoids caused further effects in knock down flies, the effects were additive, causing further fragmentation and loss of sleep. It is likely that neonicotinoid exposure caused further disruption to the electrical state of the LNvs and potentially downstream sleep promoting neurons such as the DN1s, as described above. Neonicotinoids can also cause nAChR mediated dopamine release in *Drosophila*[294]. As dopamine has a wake-promoting effect in the brain, this could contribute to the additional sleep loss and fragmentation observed in neonicotinoid exposed flies[186]. Another possibility is that the flies were experiencing starvation induced lack of sleep, as some neonicotinoid doses have been shown to reduce feeding[46,194]. However, starved flies will die after approximately 36 hours and the flies in these experiments consistently survived for over 10 days[295], showing they were eating enough to subsist on. Additionally, the reduction in feeding that can be seen in neonicotinoid exposed insects is usually attributed to appetite suppression, which wouldn't cause the food searching behaviour that results in sleep loss in starved flies[194].

In contrast to the other knock down or neonicotinoid exposed flies, exposure of Dα3 knock downs to clothianidin resulted in an increase in sleep. A potential explanation is that clothianidin is causing activation of the cholinergic, sleep-promoting subset of Kenyon cells[185] and that this effect is usually masked by its wake promoting action *via* Dα3 containing nAChR subtypes in the clock neurons.

A loss of function mutation in Dα1 had previously been shown to cause reduced night-time sleep and episode length, similar to the sleep effects observed in this chapter for the Dα1 knock down flies[85].

4.5.4 The effect of neonicotinoid exposure or nAChR subunit knock down on the circadian plasticity of the s-LNv dorsal terminals

The circadian plasticity and PDF accumulation in the s-LNv dorsal terminals were studied to: 1) Indicate whether the neonicotinoids may be acting directly upon the s-LNvs, as suspected from their effect on rhythmicity in constant conditions, for which the s-LNvs are important. 2) To investigate whether communication between the s-LNvs and downstream clock neurons such as the DN1s may be interrupted by neonicotinoid exposure. 3) To give an indication of the electrical state of the LNvs, as changes in electrical state could explain the changes in rhythmicity and sleep behaviour that have been observed.

4.5.5 Neonicotinoid exposure reduced circadian remodelling and PDF cycling

Exposure of flies to neonicotinoids caused their s-LNv dorsal terminals to stop remodelling over the 24 hour period. Unlike in control flies, whose terminals shifted from an open, highly branched structure in the day to a closed, less branched structure at night[147], neonicotinoid exposure caused these terminals to stay open and branched in both the day and the night. As the s-LNvs appear to receive excitatory ACh signalling from the HB eyelets, it is possible that the neonicotinoids are acting as agonists at the synapse between the HB eyelets and the s-LNvs, mirroring light input into the circuit and causing excitation of the s-LNvs. This could explain the constant daytime like axonal branching structure observed for the s-LNv dorsal terminals in neonicotinoid exposed flies.

In wildtype flies, PDF accumulation cycles throughout the day, with accumulation of the neuropeptide in the dorsal terminals being high in the day and low at night[147]. This change in accumulation is thought to be due to the daily change in the electrical state of the LNvs influencing the production, transportation or release of PDF[165]. PDF signalling from the LNvs is vital for both the synchronisation of the clock and for rhythmic output[142,143]. In flies who were exposed to neonicotinoids, the cycling in PDF accumulation stopped. This has been observed in flies with

hyperexcitation of the LNvs[165], which along with the arborisation data above, suggests that the loss of PDF cycling in the s-LNvs may be due to hyperexcitation caused by neonicotinoid exposure.

The loss of PDF cycling in the s-LNvs and the loss of day/night differences in electrical state suggested by this data could explain the disruptions to rhythmicity and sleep behaviour observed in Chapter 3. The loss of day/night differences in the electrical state of the LNvs can cause a breakdown in behavioural rhythmicity and a rundown of the molecular clock in constant conditions[165,167]. Hyper-excitation of the l-LNvs, key arousal neurons, can cause a loss and fragmentation of sleep[153]. The release of PDF by the LNvs is necessary for behavioural rhythmicity[3] and PDF is important for communication and synchronisation between the various clock cell groups[4]. In particular, PDF signalling is important for the activation by the s-LNvs of the DN1s, which are required for rhythmic behavioural output and which also contain a subset of key sleep promoting neurons[159,192,193,289]. This provides a potential mechanism of action for the effects of neonicotinoids on the clock and sleep in *Drosophila*.

4.5.6 *RNAi* mediated knock down of Dα1 or Dβ2 in the LNvs reduced circadian remodelling and PDF cycling

The knock down of Dα1 or Dβ2 in the LNvs also stopped the circadian remodelling of the s-LNv dorsal terminals. However, unlike for the neonicotinoid exposed flies, the terminals of the knock down flies remained in a closed, less branched form round the clock, resembling the night-time form of wildtype flies. This resembles the axonal branching structure of terminals in flies in which the LNvs have been electrically silenced[151]. This suggests that the removal of these subunits caused the loss of or reduced efficacy of nAChRs, reducing the potential for excitation of the LNvs. This electrical silencing of the s-LNvs, the key pacemaker cells of the insect brain, could explain the loss of rhythmicity in knockdown flies[151]. Potentially this silencing also occurred in the l-LNvs, which are an important part of the sleep-wake circuitry and this could contribute to the changes in sleep behaviour which was seen in knock down flies[153].

Knock down of either subunit also prevents the cycling of PDF in the s-LNv dorsal terminals. As mentioned above, the circadian cycling of PDF accumulation appears to be dependent on circadian changes in the neurons electrical state. Flies whose LNvs have been electrically silenced show a loss in cycling of PDF accumulation[151]. This further suggests that the s-LNvs may be electrically silenced by the subunit knock downs. PDF signalling is necessary for rhythmic behaviour and for signalling to downstream clock neurons such as the DN1s, and so the loss of PDF signalling may explain the reduced rhythmicity and the loss and fragmentation to sleep behaviour observed for subunit knock down flies[142,143,159,192,193,289].

4.6 Conclusions:

-The Dα1, Dα3 and Dβ2 subunits occur in the clock neurons of the fly and appear to be involved in normal sleep and circadian behaviour.

-Loss of the Dα1, Dα3 or Dβ2 subunits in the clock neurons causes reductions in rhythmicity and sleep similar to that seen in flies exposed to neonicotinoids.

-Exposure of these knock down flies to neonicotinoids does not cause any further effect on rhythmicity, suggesting that neonicotinoids affect rhythmicity directly *via* these subunits in the clock neurons.

-Exposure of knock down flies to neonicotinoids does cause further changes to sleep behaviour, suggesting that there are a variety of nAChR subtypes involved in sleep behaviour in the clock neurons.

-Neonicotinoid exposure prevents the circadian remodelling and PDF cycling of the s-LNv dorsal terminals, causing the terminals to maintain a branched, daytime like structure constantly, suggesting constant excitation.

-Knock down of Dα1 or Dβ2 has the opposite effect, causing the terminals to maintain an unbranched, night-time like structure constantly, suggesting reduced excitation.

5

Chapter 5: Imidacloprid disrupts circadian rhythmicity and sleep in *B. terrestris* foragers

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In this chapter the effects of the most commonly used neonicotinoid, imidacloprid, were tested on the locomotor and foraging rhythmicity of an important European pollinator, the buff-tailed bumblebee *Bombus terrestris*. Section 5.2 and 5.3 demonstrate the effect of imidacloprid on the locomotor rhythmicity and sleep of isolated bumblebee foragers. Section 5.4 shows the effects of imidacloprid on the foraging rhythmicity of *B. terrestris* within the full colony environment. In section 5.5 these effects are discussed, and section 5.6 provides a summary of the findings.

5.1 Introduction

Bumblebees are a diverse and important group of pollinators. In the UK alone there are 25 species of bumblebee[296] and globally there exist over 250 different species[297]. Bumblebees are major pollinators of both crops and wildflowers; of the 5 most important crop pollinators in Europe, 3 are species of bumblebee[298]. Many crops are particularly reliant on bumblebee pollination, for example soft fruits like raspberries[299] and plants that require buzz pollination, such as tomatoes[300]. Crop pollination in Europe is worth over 22 billion euros per annum and is essential to food security[298].

Unfortunately, despite their ecological and economic value, bumblebees face dramatic declines in population, with 45.6% of species in Europe in decline and 24% threatened with extinction[298]. In the UK, two species, Cullum's bumblebee (*B. cullumanus*) and the apple bumblebee (*B. pomorum*), have already disappeared and a third, the short haired bumblebee (*B. subterraneus*), had to be recently reintroduced using queens brought over from Sweden[301].

Neonicotinoid pesticides are thought to be a major factor in these declines[302], alongside parasites[48] and land use change[303]. Currently, the large majority of research into the effects of neonicotinoids on pollinators has been carried out using the honeybee, *Apis mellifera*. However, honeybees and bumblebees show differential responses to neonicotinoids, with bumblebees appearing more susceptible to lethal and sublethal effects[304]. This shows the importance of increasing the diversity of

pollinators studied in order to achieve a full picture of the ecological consequences of neonicotinoid use.

In addition to being underrepresented in the literature, bumblebees are particularly well suited for use in circadian experiments. Unlike in honeybees, the full colony and foraging arena is small enough to be contained in an incubator, allowing full control of *zeitgebers* like light and temperature, whilst allowing the social environment and foraging behaviour to be maintained. These benefits were utilised in this chapter to assess the effects of imidacloprid on forager rhythmicity and activity both in isolation and within the colony environment. This provides insight into the potential effects of neonicotinoids on foraging rhythmicity in the field, for *B. terrestris* and other pollinators.

5.2 Imidacloprid disrupted locomotor rhythmicity in isolated foragers

B. terrestris foragers who were isolated from the colony and monitored in individual tubes using the Locomotor Activity Monitor (LAM) showed rhythmic behaviour in both light:dark (LD) conditions and constant darkness (DD). There was some difference visible in locomotor behaviour for control foragers in LD compared to DD conditions. There was a notable decrease in daytime activity (t_{36}=4.7, p≤0.001) but not in night time activity (t_{36}=1.9, p=0.062) for foragers in DD. Rhythmicity also appears to decrease in DD, although this effect was not significant (t_{45}=1.8, p=0.080), however the proportion of the population experiencing arrhythmicity did increase in DD (χ^2_1=24, p≤0.001). These effects are discussed in conjunction with the effects observed for imidacloprid exposure in 5.5.1.

Exposure to imidacloprid disrupted the rhythmicity and quantity of locomotor activity (Fig. 5.1-2). In LD conditions, 10 μg/L of imidacloprid caused a decrease in mean rhythmicity (Fig. 5.1C) and both 1 and 10 μg/L caused an increase in the proportion of the population that were arrhythmic (Fig. 5.1B), from 10% in control foragers to 36% and 67% respectively (Fig. 5.1). Imidacloprid also reduced the total activity of foragers, with 1 μg/L reducing activity during both day and night and 10 μg/L reducing daytime activity (Fig. 5.1D).

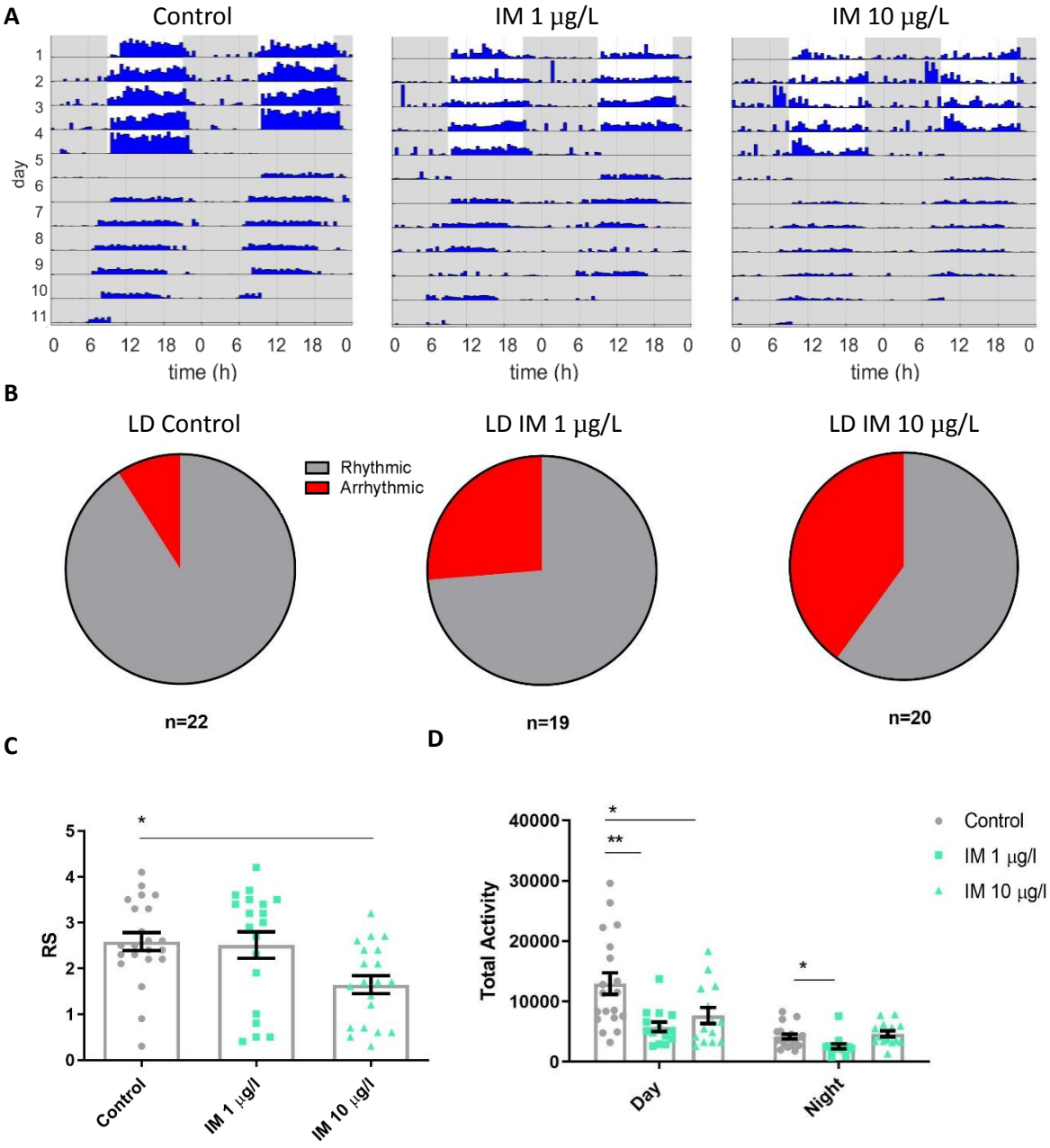

Figure 5.1 Imidacloprid affects rhythmicity in isolated *B. terrestris* foragers in light:dark

A) Representative actograms for a forager on control food, or food containing 1 µg/L or 10 µg/L of imidacloprid (IM), **B)** Proportion of foragers that were arrhythmic (RS≤1.5) in LD compared to controls for 1 µg/L (χ^2_1=10.0, p=0.002) and 10 µg/L (χ^2_1=25.6, p≤0.001), **C)** Mean rhythmicity for either control foragers or those fed 1 or 10 µg/L, in LD conditions ($F_{2,58}$=5.3, p=0.008), **D)** Total activity for foragers in each treatment group in LD conditions, during the day ($F_{2,44}$=6.7, p=0.003) and the night ($F_{2,44}$=5.4, p=0.008). Each data point represents a single bee, n=19-22 bees for each treatment group.

Conversely, in DD, exposure to either dose of imidacloprid had little effect on foragers' activity or rhythmicity (Fig. 5.2). Foragers fed 1 or 10 μg/L of imidacloprid had the same mean rhythmicity and levels of activity as control foragers (Fig. 5.2B-C). The proportion of each population that were arrhythmic was also similar, with 40% of control foragers experiencing arrhythmia compared to 33% at 1 μg/L of imidacloprid and an increase to 50% at 10 μg/L (Fig. 5.2A).

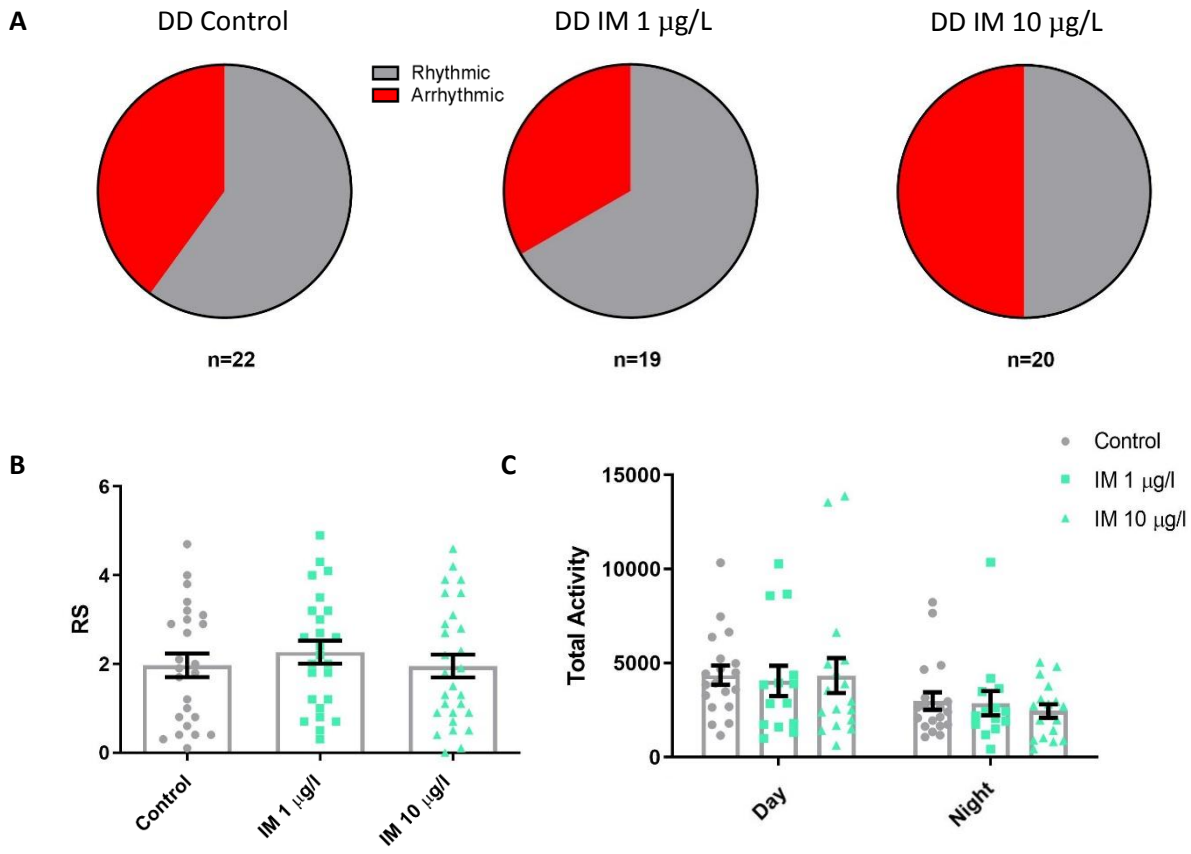

Figure 5.2 Imidacloprid doesn't affect rhythmicity in isolated *B. terrestris* foragers in constant darkness

A) Proportion of foragers that were arrhythmic (RS≤1.5), compared to controls, in DD for foragers on IM 1 μg/L (χ^2_1=1.0, p=0.33) or IM 10 μg/L (χ^2_1=2.0, p=0.16), **B)** Mean rhythmicity for foragers in each treatment group in DD conditions ($F_{2,47}$=0.5, p=0.637), **C)** Mean activity for foragers in each treatment group in DD conditions, during the subjective day ($F_{2,47}$=0.1, p=0.947) and night ($F_{2,47}$=0.6, p=0.541). Each data point in the histograms represents a single bee, n=14-22 bees for each treatment.

5.3 Neonicotinoids increased sleep in isolated foragers

Foragers who were exposed to 10 μg/L of imidacloprid showed an increase in sleep compared to controls, particularly during the day (Fig. 5.3A-B). This is likely due to the increased number of daytime sleep episodes (Fig. 5.3C) initiated by these foragers. The length of these sleep episodes was the same as in control foragers (Fig. 5.3D). A dose of 1 μg/L of imidacloprid had no effect on the quantity or structure of sleep.

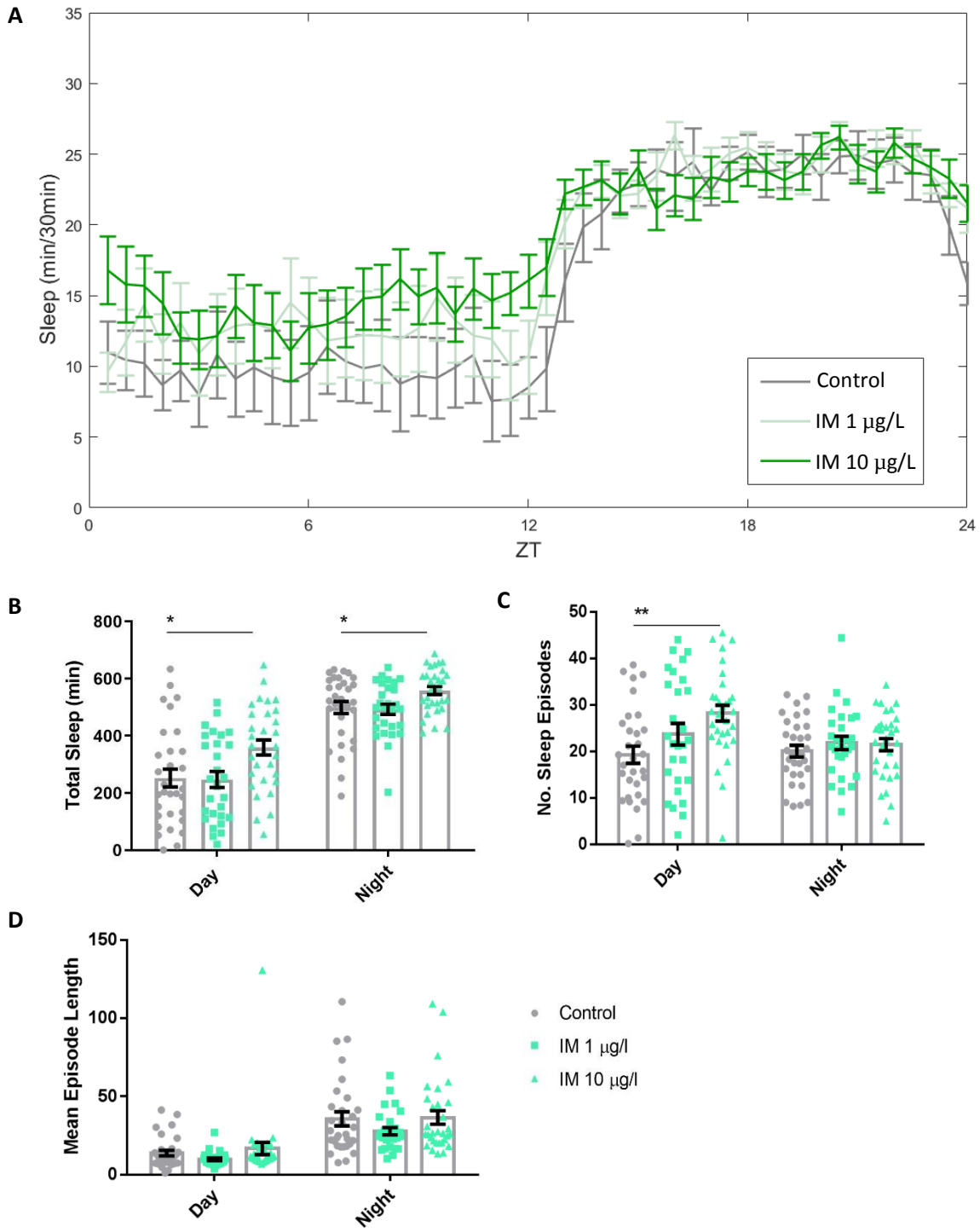

Figure 5.3 Imidacloprid increased sleep in isolated bumblebee foragers

A) Mean total sleep achieved for control foragers and those fed 1 or 10µg/L of imidacloprid (IM), per 30 min bin over the 24 hour period, **B)** Mean total sleep (min) for each treatment group in the day ($F_{2,87}$=4.9, p=0.010) and the night ($F_{2,87}$=4.1, p=0.019), **C)** Mean number of sleep episodes initiated for each treatment group during the day ($F_{2,87}$=5.4, p=0.006) and the night ($F_{2,87}$=0.490, p=0.614), **D)** Mean sleep episode length for each treatment group during the day ($F_{2,87}$=1.7, p=0.182) and the night ($F_{2,87}$=1.5, p=0.238). Each data point in the histograms represents a single bee, n=28-31 bees for each treatment.

5.4 Neonicotinoids disrupted foraging rhythmicity of foragers in a full colony environment

B. terrestris foragers in a full colony setting show diurnal rhythms in foraging activity (Fig. 5.4-5). This rhythmicity was disrupted in foragers exposed to 10 μg/L of imidacloprid in both field-relevant LD light conditions (Fig. 5.4) and in constant darkness (DD), (Fig. 5.5). In LD conditions, imidacloprid caused a decrease in the mean rhythmicity of foragers (Fig. 5.4B) and caused the proportion of foragers who were arrhythmic to increase from 48% to 65% (Fig. 5.4A). Imidacloprid also caused a decrease in foraging activity for both daytime and night-time (Fig. 5.4C).

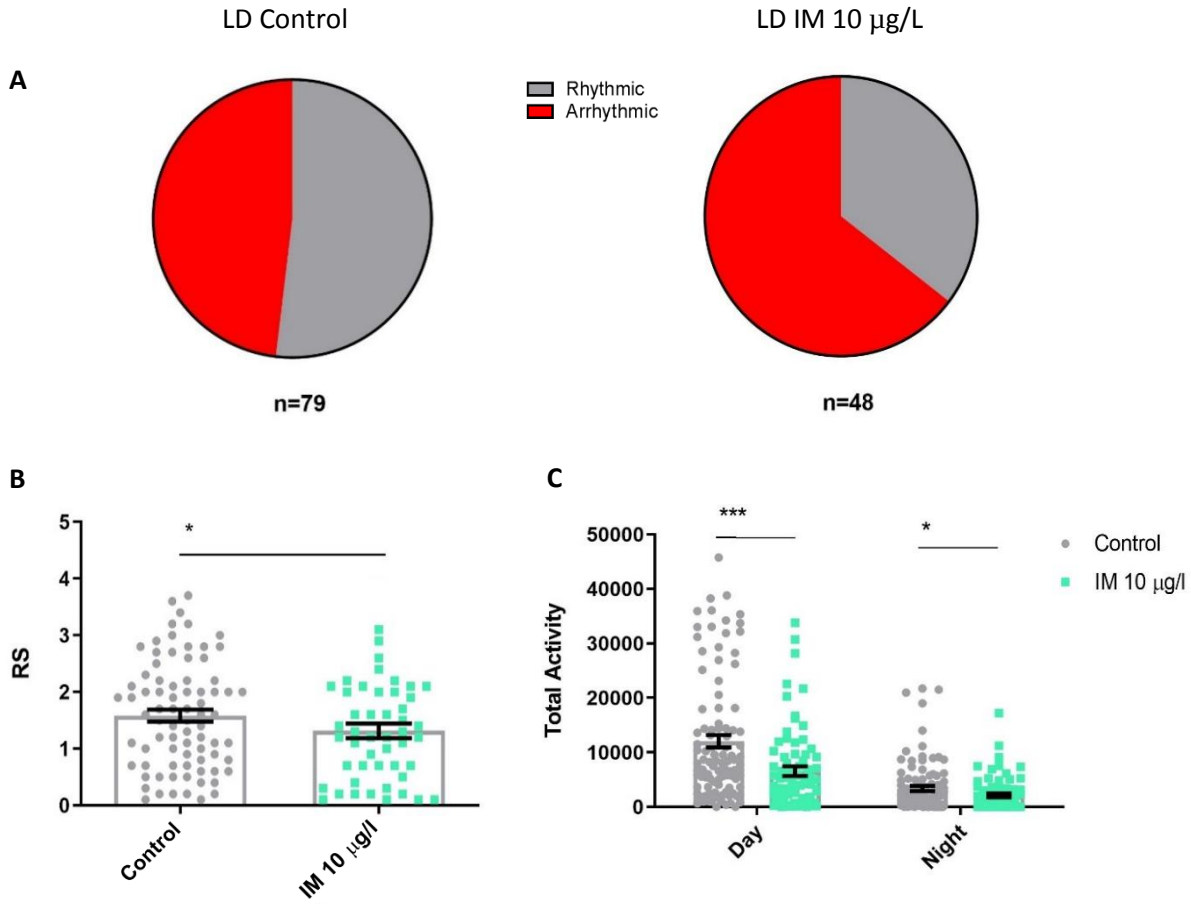

Figure 5.4 Imidacloprid reduces foraging rhythmicity and activity in bumblebee foragers in LD within the colony

A) Proportion of the population that were arrhythmic in LD for each control foragers compared to those on 10 μg/L (χ^2_1=5.26, p= 0.022), **B)** Mean rhythmicity for either control foragers or foragers fed 10 μg/L, in LD (t_{125}=2.0, p=0.048), **C)** Mean activity for foragers in each treatment group in LD, during the day (t_{170}=3.8, p<0.001) and the night (t_{170}=2.0, p=0.042). Each data point in the histograms represents a single bee, n=48-79 bees for each treatment for rhythmicity, n=74-100 bees for each treatment for activity.

In DD conditions, imidacloprid caused a reduction in mean rhythmicity for foragers (Fig. 5.5C) and an increase in foraging activity during the subjective night (Fig. 5.5D). The proportion of foragers that were arrhythmic for control and imidacloprid exposed bees were similar in DD, 31% and 36% respectively (Fig. 5.5B).

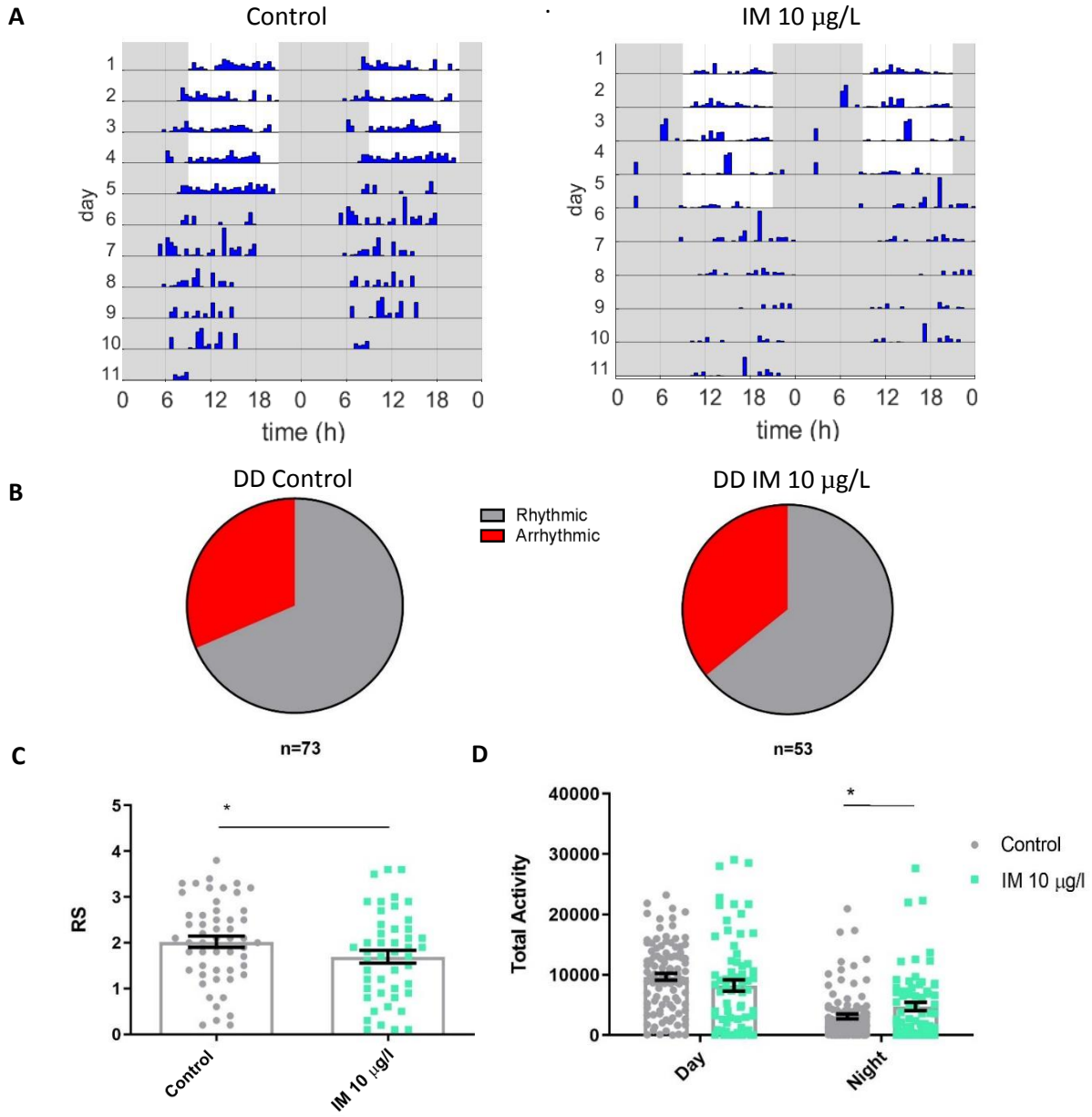

Figure 5.5 Imidacloprid reduces foraging rhythmicity and activity in bumblebee foragers in DD within the colony

A) Representative actograms for a forager on control food and one dosed with 10 µg/L imidacloprid (IM), (χ^2_1=0.36, p=0.550), **B)** Proportion of the population that were arrhythmic in DD for control foragers compared to those on 10 µg/L, **C)** Mean rhythmicity for foragers in each treatment group in DD conditions (t_{125}=2.2, p=0.029), **D)** Mean activity for foragers in each treatment group in DD conditions, during the subjective day (t_{169}=1.6, p=0.105) and night (t_{114}=-2.0, p=0.043). Each data point in the histograms represents a single bee, n=48-79 bees for each treatment for rhythmicity, n=74-100 bees for each treatment for activity.

5.5 Discussion

5.5.1 Neonicotinoids disrupted locomotor rhythmicity in isolated foragers

Isolated foragers showed circadian rhythmicity in locomotor behaviour in both LD and DD conditions, as has been previously shown in *B. terrestris*[211]. Imidacloprid caused a decrease in this rhythmicity and reduced activity in LD light conditions. Once placed into constant conditions, the differences in rhythmicity and activity between imidacloprid exposed foragers and controls were lost. This was due to control foragers becoming less active and having a higher proportion of the population that were arrhythmic once light cues were removed. This may suggest that the imidacloprid interfered with light sensing or entrainment, causing imidacloprid exposed foragers to have activity levels and rhythmicity in LD resembling that of control foragers in constant darkness. Work carried out in *Drosophila* has shown that the communication between the light sensing organs and the PDF expressing, pacemaker neurons of the clock utilises ACh and is reliant on nAChRs[90,91]. Whilst this pathway has not yet been shown in bees, the bee central clock, which is best characterised in the honeybee, appears to closely resemble that of *Drosophila*. They possess clock neurons which express both *per* and PDF, and in which the accumulation of PDF is under circadian control[225]. PDF release in the honeybee brain effects the phase of the clock and circadian outputs, like in flies, suggesting that these PDF+ neurons are the pacemaker neurons of the clock in bees, as in flies. These PDF+ clock neurons project to many brain regions including towards other potential clock neuron groups and extensively towards the visual organs[225]. The PDF+ clock neurons of honeybees also project to the *pars intercerebralis* and *pars lateralis*[225] which are key areas for control of locomotor activity and sleep in *Drosophila*[159,187,188,225]. Whilst the clock of *B. terrestris* is less well characterised, they appear to have the same number of neurons expressing both per and PDF, in the same location in the brain, as has been seen in honeybees[226]. PDF+ pacemaker neurons with similarly extensive branching patterns have also been identified in other insects, including crickets and cockroaches, suggesting that the circuitry of the central clock is well conserved within insects. This may mean that the PDF+ neurons in bumblebees receive excitatory ACh signalling from the visual circuit as is seen in flies. The PDF neurons in honeybees have projections into every optic ganglia in the bee brain such as the medulla, lamina, lobula and also to the ocelli[225] and acetylcholine is the predominant excitatory neurotransmitter in the insect CNS[305]. If the PDF+ clock neurons do receive nicotinic input from the light sensing organs in *B. terrestris*, imidacloprid may be acting as an agonist at these synapses, disrupting the signal from the light sensing organs and preventing the normal function of the clock neurons. The lack of a light sensitive cry may also make bees more susceptible to disruption of the clock by neonicotinoids as they may be more dependent on the visual system and clock circuitry for setting the pace of the clock[218].

5.5.2 Neonicotinoids increased sleep in isolated foragers

Under LD conditions imidacloprid also caused a reduction of locomotive activity in day and night and an increase in the number of daytime sleep episodes that isolated foragers experienced. Exposure to neonicotinoids has previously been shown to reduce locomotor activity in bees. Imidacloprid has been shown to reduce locomotion in isolated *B. terrestris*[306] and in other pollinators such as the sweat bee *Melipona quadrifasciata anthidioides*[45] and solitary bees like *Osmia bircornis*[307]. Previous work in other insects has shown that neonicotinoids can act directly on the thoracic ganglia which control motor function in insects[24] so neonicotinoids may be acting directly through these ganglia, causing activation and or depolarisation block which compromises locomotion. Neonicotinoid exposure also appear to change the expression of hundreds of genes in worker bees, including genes which are involved in locomotion, such as titin[308], providing another possible explanation for the reduced activity observed. Alternatively, the reduction in daytime activity and the increase in daytime sleep episodes may be due to the disruption in circadian rhythmicity. As mentioned above the PDF+ neurons which set the pace of the clock and thus of activity and sleep, are nicotinic in *Drosophila*[90,91]. These neurons project to brain regions involved in sleep and activity like the *pars intercerebralis* and *pars lateralis*[159,187,188,225]. If these neurons are nicotinic in bees, then imidacloprid may be acting on them which could disrupt the normal timing of sleep and activity in diurnal foragers. Another group of neurons that imidacloprid could be acting upon are the Kenyon cells of the mushroom body. It has been shown in honeybees and bumblebees that many of the Kenyon cells are susceptible to neonicotinoids, including imidacloprid[75,232]. The mushroom body has been shown to be involved in sleep in *Drosophila*[183], with sleep-promoting mushroom body output neurons utilising acetylcholine signalling[185]. Imidacloprid could be acting upon these cells, activating them and resulting in the general increase in sleep observed for exposed bees.

5.5.3 Neonicotinoids disrupted foraging rhythmicity of foragers in a full colony environment

Foragers who were in the full colony environment showed a similar disruption of the clock when exposed to imidacloprid as seen in isolated foragers. Within the colony setting, imidacloprid caused reduced rhythmicity in foraging activity in both LD conditions and in constant darkness. Where isolated control foragers experienced a reduction in rhythmicity once light cues were removed, resulting in their rhythmicity matching that of imidacloprid exposed foragers in DD, control foragers in the full colony setting maintained similar levels of rhythmicity in both LD and DD. This strong colony rhythmicity in constant conditions has been seen before[202] and is likely due in part to the strong *zeitgeber* that the social environment of the colony provides[208].

The reduction of foraging rhythmicity observed in imidacloprid exposed bees provides further evidence that imidacloprid disrupts the clock in bumblebees, potentially through the mechanisms suggested in the discussion of the clock in isolated foragers, in section 5.5.1. It also suggests that the social environment of the colony cannot mitigate the effects of imidacloprid on rhythmicity.

Under field-relevant LD conditions, the quantity of foraging activity was reduced for imidacloprid exposed colonies. This effect has been reported before. Another study looking at *B. terrestris* colonies in semi-field conditions found that imidacloprid exposure resulted in a decrease in the number of foraging trips carried out by foragers[309], to the point where the colony began producing extra foragers in an attempt to mitigate this. As a result, exposure to neonicotinoids has been shown to reduce the quantity of sucrose and pollen collected by *B. terrestris* colonies[310]. Imidacloprid also appears to reduce general activity levels within the colony. One study showed that 6 µg/L of imidacloprid caused 'lethargy', reducing activity within the colony including brood care and social interactions[311]. The effects observed here on foraging activity may be part of this general reduction in activity. They could also be driven by a loss of appetite. Reduced sugar consumption caused by neonicotinoid exposure has been observed in *B. terrestris*[28] and other pollinators such as honeybees[312] and the solitary bee *O. bicornis*[307]. In fact, Cresswell *et al*. found that a dose of 10 µg/L, the dose used in this chapter, could cause a decrease in feeding of 30% in *B. terrestris*[304] This appetite suppression is likely to reduce foraging motivation.

In DD conditions, the activity levels of imidacloprid exposed foragers were similarly low in the day but they were slightly more active at night than control foragers, providing further evidence of a disruption to the clock.

5.5.4 Possible consequences in the field

The reduction in foraging activity and rhythmicity observed for bumblebee colonies exposed to imidacloprid is likely to have deleterious consequences in the field. The clock is vital to foraging, as many aspects of floral resources are under circadian control[11]. A disruption to the clock in foragers may reduce their foraging efficiency as they will be less able to form the time-memories required to accurately visit different flowers[196]. The clock also feeds into the sun-compass navigation pathway that foragers use to navigate[225]. Neonicotinoids have previously been shown to reduce homing ability in honeybees[278] and disruption to the clock could be a contributing factor to this. Reduced foraging efficiency is likely to conflate the reductions in appetite and foraging motivation that neonicotinoids cause and reduce the capacity of the colony to grow and reproduce. Reduced feeding and foraging are associated with less brood production[28] and smaller colonies are less resilient and less likely to produce queens[41].

Queens themselves represent a particularly vulnerable stage in the life cycle of the colony. When the bumblebee queen comes out of hibernation and initiates a new colony, she must forage for herself and the first generation of workers. Disruption to her foraging activity and rhythmicity during this period could reduce the likelihood of her successfully initiating the colony. Reduced feeding has already been observed for queens caught from the wild post-hibernation and fed field relevant doses of neonicotinoids[313]. Indeed, imidacloprid exposure of 5ppb can cause queen *B. terrestris* to become less active, delay initiation of the nest and produce less brood[314]. Bumblebee queens also appear to be more susceptible to neonicotinoids than workers, as shown in *B. impatiens*[308]. Additionally, they may also be more likely to encounter effective doses. *B. terrestris* queens hibernate in the soil, where neonicotinoid concentrations can be up to 60 times higher than in nectar or pollen[315], and are most active during spring when crops that are often treated with neonicotinoids, such as oil seed rape, tend to flower.

5.6 Conclusions

- Imidacloprid can reduce locomotor and foraging circadian rhythmicity in bumblebee foragers
- Imidacloprid reduces locomotor and foraging activity in foragers, particularly in daytime

6

Chapter 6: Discussion

As introduced in Chapter 1, neonicotinoids are the most commonly used insecticides in the world[23]. They are pervasive, persisting in the environment long after their initial use and can cause a large range of sub-lethal effects to beneficial insects through their action as an agonist at nAChRs[24,26,30,36,38-40]. The nAChRs continue to be a popular target site for novel pesticides, making research into the adverse effects of the modulation by neonicotinoids on beneficial insects such as pollinators important[68].

6.1 Neonicotinoids disrupt circadian rhythmicity and sleep

In this thesis, the effects of neonicotinoids on the circadian rhythms and sleep of insects were thoroughly characterised for the first time. *Drosophila* was used as a model insect to allow rapid and extensive investigation of the behavioural effects and mode of action of a range of neonicotinoids. Imidacloprid, clothianidin and thiamethoxam, the three most widely used neonicotinoids and those currently covered by the EU ban[58], were all found to reduce behavioural rhythmicity and fragment sleep at field relevant doses in *Drosophila*. Flies were less rhythmic in both LD conditions and constant darkness. Their sleep consisted of frequent short sleep episodes, and the total quantity of sleep achieved was reduced. These effects were observed for doses of neonicotinoid that might commonly be found in the nectar of treated plants[19], suggesting that disruption to sleep and the circadian clock may be sub-lethal effects experienced by non-target insect species in the field. Thiacloprid, one of the two neonicotinoids still currently used in the EU[59], had no effect on rhythmicity. This is in line with the lower lethality observed for thiacloprid, which has an LD_{50} over 24 hours of 24 ng per honeybee compared to 18 ng for imidacloprid[265], and in our own work in the lab (Appendix 1). However, thiacloprid did significantly disrupt sleep behaviour in *Drosophila* at field-relevant doses, suggesting that its continued use should be considered carefully when voted on by EU members at the end of 2019.

Behavioural assays were repeated in *B. terrestris* and showed comparable results for the effects of field relevant doses of imidacloprid on forager's circadian rhythms. Foragers of *B. terrestris* showed

reduced locomotor rhythmicity when in isolation and reduced foraging rhythmicity when free flying within the colony. This reduced rhythmicity was observed in both LD and constant conditions. Both isolated foragers and foragers within the colony also showed a dramatic reduction in activity when exposed to imidacloprid and isolated foragers appeared to sleep more during the day, with longer sleep episodes.

6.2 The effects of neonicotinoids on sleep and the validity of *Drosophila* as a model

Neonicotinoids were shown to consistently fragment and reduce sleep in *Drosophila*. Deep sleep is important for memory consolidation in *Drosophila* and bees[106,176], with sleep deprivation reducing the ability of honeybees to learn navigational routes[16]. Deep sleep occurs later in the sleep episode[173], so the fragmentation of sleep observed in neonicotinoid-exposed *Drosophila* is likely to reduce the quantity of deep sleep achieved. However, the effect of neonicotinoids on sleep observed in *Drosophila* was not replicated in *B. terrestris*. Imidacloprid exposure in isolated *B. terrestris* foragers resulted in an increase in day-time sleep, with longer sleep episodes. It is possible that modulation of sleep occurs differently in bees than in flies. Currently, the brain regions and pathways that modulate sleep in bees are unknown making it difficult to compare.

Alternatively, this difference could represent the greater effect that imidacloprid exposure had on activity levels in *B. terrestris* compared to *Drosophila*. Due to the use of beam crosses as a metric of activity, the *Drosophila* or locomotor activity monitor (DAM or LAM) equipment cannot distinguish between inactivity and sleep. Imidacloprid exposure caused activity to halve in isolated foragers in LD conditions at all doses, compared to *Drosophila* in which it caused no consistent change to activity. Imidacloprid has previously been shown to reduce overall activity within the colony in *B. terrestris*, reducing foraging, brood care and social interactions[311]. This 'lethargy', as one author described it, could be interpreted as sleep by the LAM equipment, meaning that the possibility that foragers are experiencing both reduced activity and fragmentation of sleep should not be discounted, given the detrimental implications this could have for learning and memory. There are ways of more accurately identifying sleep in foragers. Sleep is associated with specific postural changes in honeybees, which can be identified using video tracking[316]. Honeybees also exhibit sleep stage specific antennal movement, reminiscent of Rapid Eye Movement (REM) in humans, which allow deep sleep to be recognised[106]. These sleep indicators may also occur in bumblebees and could be used to quantify the effect of neonicotinoids on the quantity of sleep and deep sleep achieved by foragers. Alternatively, the *Drosophila* Arousal Tracking (DART) system, which was designed to measure sleep stage through quantifying arousal threshold in *Drosophila*, could be adapted for use with bumblebees[168].

Additionally, sleep was only measured in isolated foragers. Whilst the effects of imidacloprid on the activity and rhythmicity of foragers in isolation and the colony appear to be the same, the colony environment does affect behaviour. For example, control foragers experienced a decrease in rhythmicity in constant conditions when isolated but not when they were in the colony. Sleep has also been shown to be influenced by social cues within the colony, such as the presence of brood[317]. The radio frequency ID (RFID) system used to measure foraging rhythmicity in the colony does not allow sleep to be quantified, as it only monitors the entrance and exit of foragers into the colony. However, there are means of assessing sleep within the colony environment that could be explored, such as the Behavioural Ecology tag (BEETag) system[318], which uses digital barcodes (specifically, QR codes) in the place of RFID tags. A camera can then be placed inside the colony and the movement and placement of foragers within the colony can be monitored. Foragers tend to occupy specific regions of the colony whilst sleeping so this may allow an estimation of sleep within the colony environment[319].

It would also be interesting to repeat the rhythmicity and activity assays in *B. terrestris* for clothianidin, which unlike imidacloprid, consistently caused hyperactivity in *Drosophila*, as well as having greater effects on rhythmicity and sleep. Another interesting avenue for future work would be to explore whether the effects of neonicotinoids on sleep in *Drosophila* are sufficient to explain the effects of neonicotinoids on other behaviours such as learning and memory. Many of the sub-lethal behavioural effects already observed for neonicotinoids are behaviours that are influenced by the clock or sleep, for example, longevity, pheromone communication and learning and memory[13,96,176,287,320,321]. It is possible that the disruption to the clock and sleep by neonicotinoids observed in *Drosophila* could be a contributary factor or even causal to the other behavioural and sub-lethal effects of the neonicotinoids. To test this hypothesis, future work could test behaviours like learning and memory for flies with knock down of Dα1, Dα3 or Dβ2 in the clock neurons. These knockdowns mimic the sleep and circadian effects of neonicotinoids, without exposing the insect to the drug. This would allow the effects of neonicotinoid susceptible nAChR mediated sleep and circadian disruptions on other behaviours like learning to be tested. Alternatively, flies could be exposed to neonicotinoids and then deep sleep could be pharmacologically or genetically induced to see whether this rescued learning[322]. These sorts of experiments could provide insight into the interconnected and synergistic nature of sub-lethal effects.

6.3 The effects of neonicotinoids on the insect clock can be modelled in *Drosophila*

The effects of neonicotinoids on circadian rhythmicity in both species were very similar. In both *Drosophila* and *B. terrestris*, exposure to imidacloprid reduced mean rhythmicity and increased the

proportion of individuals that were behaviourally arrhythmic, in both LD and DD conditions. This suggests that *Drosophila* may represent a good model for exploring the effects of neonicotinoids and other nAChR targeting insecticides on the insect clock. The circuitry of the central clock appears to be well conserved in insects. *Drosophila* and the honeybee both possess PDF+ pacemaker neurons that project to other clock neurons and the light sensing organs, and which exhibit day night changes in PDF accumulation[90,91,147,159,225]. The bumblebee *B. terrestris* contains PDF+ neurons of the same number and location as in honeybees and PDF+ pacemaker neurons with similarly extensive branching patterns have been identified in other insects including crickets and cockroaches[226,323]. The effect that neonicotinoid exposure had on circadian remodelling and PDF cycling of the s-LNv dorsal terminals of *Drosophila* shows that neonicotinoids can affect circadian outputs in the PDF+ neurons themselves. Due to the conserved nature of the PDF+ neurons, this could explain the disruption to behavioural rhythmicity seen in *B. terrestris* foragers and may suggest that neonicotinoid exposure could disrupt rhythmicity in insects beyond *Drosophila* and *B. terrestris*.

This is not the first example of neonicotinoid sub-lethal effects being identified for both *Drosophila* and the bee. Neonicotinoids have been shown to affect longevity[324], the immune system[325], and learning and memory in *Drosophila* (Appendix 1), all of which have been identified as sub-lethal effects in bees[267,287,315]. Replicating sub-lethal effects in *Drosophila* allows the assays and genetic tools available in *Drosophila* to be used to explore the mechanisms by which neonicotinoids disrupt behaviours. This dual approach can be very effective. For example, neonicotinoids have been shown to reduce the immune response in honeybees and increase their vulnerability to pests[40]. Recent work in *Drosophila* has identified a potential pathway for this effect, showing that neonicotinoid exposure caused an increase in the transcription of a gene which inhibits NF-kB immune signalling, reducing antiviral defences in the insect[326]. Identifying the specific mechanisms through which neonicotinoids disrupt behaviour in the insect allows us to build a more comprehensive understanding of its effects in the body, other potential off-target effects that may be occurring as well as the function of nAChRs in healthy insects, hopefully informing the design of safer insecticides in the future.

The work carried out in this thesis provides another example of this comparative approach. Here, novel sub-lethal effects on circadian rhythmicity and sleep were identified, due to knowledge of the importance of ACh signalling to these behaviours in *Drosophila*[88,90-92,153,231,232]. Disruptions to these behaviours were then identified in a pollinator *B. terrestris* and further work in *Drosophila* allowed a potential mechanism of action to be identified. This work also provided novel information about the bumblebee clock, suggesting the involvement of ACh signalling/ nAChRs in the normal function of the central clock.

Identification of sublethal effects that occur in both *Drosophila* and pollinators also provides the opportunity to use *Drosophila* to carry out rapid screening of novel nAChR targeting insecticides[68] before they enter the market/ field.

6.4 Towards a mechanism of action for the effects of neonicotinoids on the insect clock

After identifying the behavioural effects of neonicotinoids on circadian rhythmicity and sleep, further work was carried out in *Drosophila* towards identifying a mechanism of action. Utilising the genetic tractability of *Drosophila,* mutants where created with *RNAi* mediated knock down of neonicotinoid susceptible nAChR subunits within the clock neurons. The results from these experiments suggest that neonicotinoids disrupt the insect clock through action *via* nAChRs containing Dα1, Dα3 and Dβ2 within the clock neurons of the brain. Knock down of these subunits in the clock neurons caused disruptions to rhythmicity and sleep matching those observed in neonicotinoid exposed flies. This suggests that Dα1, Dα3 and Dβ2 all play a role in normal sleep and circadian behaviour in the fly and occur in the clock neurons. Loss of function mutations in Dα1 has previously been shown to cause loss of sleep similar to that shown in this thesis[85,86], providing further evidence for a role of this subunit in sleep maintenance. This is the first time that Dβ2 has been implicated in sleep or that a role for any subunit in the insect circadian clock has been identified, though the importance of ACh signalling to the clock of *Drosophila* has been shown previously[90-92,153,231]. The subunits Dα3, Dα6 and Dβ1 have been shown to be rhythmically expressed in the PDF+ neurons in *Drosophila*[288]. This might suggest that Dα6 and Dβ1 could also play a role in the clock and might be good candidates to explore next. The subunit Dα6 in involved in susceptibility to the insecticide spinosad[89]. Spinosad also targets the nAChRs[327], is currently used in the EU and qualifies for use in organic farming[69]. It would be valuable to assess the effects of spinosad on the clock and sleep in insects, potentially using the *Drosophila* model as carried out for neonicotinoids in this thesis.

Dα1, Dα3 and Dβ2 may be involved in neonicotinoid susceptibility in many insect species as Dα1, Dα3 and Dβ2 appear in the groups of receptors that are well conserved amongst insects, including beneficial insects such as honeybees[72]. Dα1 has 71% amino acid identity with amelα1, whilst Dα3 and Dβ2 have 70% identity with amelα3 and amelα8 respectively[328]. The amelα1, amelα3 and amelα8 also contain the F loop insertion seen in their *Drosophila* subunit counterparts and which is thought to be involved in imidacloprid susceptibility[328,329]. Thus, it seems likely that these subunits are involved in neonicotinoid susceptibility in beneficial insects such as bees and could play a similar role in receptors and behaviours.

The use of the *GAL4* mediated *RNAi* knockdown of specific nAChR subunits in behaviourally relevant neurons in *Drosophila*, *e.g.* clock neurons, could be expanded to identify further behavioural roles for nAChR subunits. This has been carried out successfully in our lab to identify a role for Dα1 and Dβ2 in learning and memory by targeted expression in the mushroom bodies, providing insight into the mode of action for neonicotinoids on learning in insects and, by extension, pollinators (Appendix 1). Identification of the behavioural role of different nAChR subunits may also aid in informing which subunits assemble into functional nAChRs in the insect. For example, the similarities observed in the effect of the knockdown of Dα1, Dα3 and Dβ2 in the clock on circadian rhythmicity may suggest that they appear in the same receptor. This is supported by the lack of additive effect on rhythmicity for these knock downs when exposed to neonicotinoids, suggesting that these appear in the only neonicotinoid susceptible receptor or receptors involved in the clock. This could be further investigated by knocking out multiple subunits in the clock neurons simultaneously and seeing whether further behavioural deficits are observed. Given the difficulty of expressing native heteromeric insect nAChRs[72], this functional analysis provides valuable information on native nAChR composition.

6.5 Role of PDF+ neurons in neonicotinoid disruption of the clock

Neonicotinoid exposure caused no further reduction in rhythmicity in nAChR subunit knockdown flies, suggesting that neonicotinoids are reducing behavioural rhythmicity through interaction with the clock neurons. As the central clock is reliant on the nicotinic PDF+ clock neurons[90,151], neonicotinoids may be acting via these neurons to cause the circadian disruptions observed. This theory is supported by the finding that neonicotinoid exposure affects the circadian remodelling of the PDF+ s-LNv neuron dorsal terminals. In neonicotinoid exposed flies, these terminals possessed a branched, open structure at all times, resembling that of control terminals in daytime, when they are receiving excitatory ACh input from the light sensing organs[147]. This could suggest that the neonicotinoids may be acting to excite the PDF+ neurons. Knockdown of Dα1, Dα3 and Dβ2 caused the terminals to have a simple, night-time like structure at all times, suggesting that knockdown of these neonicotinoid susceptible subunits and the receptor/s they form reduced excitation of the PDF+ neurons[151]. The cycling of PDF within these terminals, whose circadian release is thought to be reliant on day-night differences in the electrical state of the PDF+ neurons[151,165], was prevented by both neonicotinoid exposure and nAChR subunit knockdown. PDF signalling is required for communication and synchronisation within the clock neurons and for rhythmic behavioural output, so this could explain the reduction of behavioural rhythmicity observed in neonicotinoid exposed flies[142,143]. The expression of PDF is also important for the occurrence and timing of other behaviours

in the animal, for example in sex pheromone production and mating timing and quantity[13], suggesting that disruption to these clock neurons could have far reaching behavioural effects.

Given that the PDF+ neurons are also the pacemaker cells of the honeybee and bumblebee central clock[225,226], disruptions in PDF signalling could explain the reduction in behavioural rhythmicity observed for *B. terrestris*. As in flies, the PDF+ neurons of bees project to many brain regions involved in behaviours which are influenced by the clock, suggesting that neonicotinoid induced disruption of PDF+ signalling could have wide reaching effects[225]. The PDF+ neurons of honeybees extend into areas involved in the sun-compass pathway, such as the dorsal rim area of the lamina and the medulla[225]. This dorsal rim area is important for sensing polarised light, used by many bees for navigation, and in honeybees for communication via the waggle dance[330]. It is thought that the proximity of the clock neurons to this sun-compass pathway may allow the bee to integrate time and place information[225]. The PDF+ neurons also extend to the mushroom bodies[225], which are the important for learning and memory in the insect[182]. Learning in bees is influenced by the clock, with honeybees learning better in the morning and being capable of impressive feats of time-memory, such as learning up to nine different time specific feeders[196,199]. This could be mediated by the PDF+ projections to the mushroom body[225]. If neonicotinoids can act directly upon the PDF+ neurons of the insect clock, as the results in *Drosophila* might suggest, then disruption to the clock could also disrupt behaviours which the clock influences in bees, such as learning, navigation and communication. Neonicotinoids have already been shown to have a detrimental effect on the learning and navigational capacity of honeybees and bumblebees and disruption to the clock could be a contributing factor to this[278,287,331].

6.6 Disruption of the clock in bumblebees

Previous work on the bee clock and on neonicotinoid sub-lethal effects has tended to focus on honeybees. Given the differences observed in neonicotinoid susceptibility between honeybees and other species of bee it is important to increase the diversity of species studied[304]. Studying the clock in *B. terrestris* also allows the impact of the full colony environment on the clock to be investigated in the lab[202]. In honeybees, social cues are a stronger *zeitgeber* than light[208]. However, the social environment did not mitigate the effects of neonicotinoids on the clock. This suggests that either the central clock was thoroughly disrupted by neonicotinoid exposure, preventing social cues from entraining it, and/or that pheromone communication or sensing was disrupted[321]. Additionally, this may suggest that neonicotinoids are rapidly disseminated throughout the colony, resulting in colony wide reductions in rhythmicity. Either way it appears that neonicotinoid exposure could cause significant reductions in foraging rhythmicity for bumblebee colonies in the field.

The clock is important throughout the life cycle of the colony. As shown in this thesis for *B. terrestris*, foraging activity and rhythmicity is reduced by exposure to imidacloprid, reducing daytime foraging and increasing the likelihood of inappropriate, night-time foraging activity. This may affect the ability of the queen to initiate the colony and of the colony to grow and reproduce, as only the largest colonies produce new queens[41]. In this way, neonicotinoid-induced reductions in foraging rhythmicity could have detrimental consequences for the survival of bumblebees in the field. Bumblebees may also be particularly vulnerable because they exhibit size-based division of labour, making transition between roles more difficult than in honeybees[211,237]. If foragers are removed from a bumblebee colony, only a small portion of in-nest workers are able to become rhythmic and begin foraging[65]. This means that for bumblebee colonies exposed to imidacloprid, despite reduced activity from current foragers, the colony has to wait till the next generation before being able to increase the proportion of foragers[309]. Producing more foragers is also costly, especially if those workers are likely to experience neonicotinoid exposure too. A weakening of the clock may also reduce foragers ability to adapt to changing day lengths in the field[156], causing further reductions in foraging efficiency.

6.7 Consequences for other pollinators

Given the similarity in the response of the clock in *Drosophila* and *B. terrestris*, and the conservation of the PDF+ neurons between these and other insects[141,225,226,323], neonicotinoids may also disrupt the clock in other pollinators. Due to the importance of the clock and rhythmicity this could have wide ranging impacts. Honeybees use the time input into the sun-compass to perform the waggle dance and communicate the location of resources to other foragers[12,225]. They also use the clock and assessment of photoperiod to help determine when to start rearing fresh brood in preparation for the spring[332]. At this time in the year the colony is often low on food and so mistiming this and producing brood too early or too late could be costly. Additionally, rhythmicity in honeybee workers appears to be inherited. If worker bees are not exposed to the rhythmic colony environment during their first 48 hours after eclosion, they do not appear to develop rhythmicity on their own[207]. This suggests that a loss of colony rhythmicity could result in the production of future generations of arrhythmic workers, even if those workers themselves were not exposed to neonicotinoids. Whilst circadian rhythmicity is less well studied in solitary bees, one study looking at the large carpenter bee, *Xylocopa (Proxylocopa) olivieri* found their foraging activity to be under circadian control. These bees were crepuscular, foraging at dawn and dusk, allowing them to minimise foraging competition and to dedicate the day to defending the nest[333]. Disruption of foraging rhythmicity in these bees could make the nest more vulnerable to predation.

6.8 Concluding remarks

Neonicotinoids clearly affect activity, sleep and behavioural rhythmicity in *Drosophila* and *B. terrestris*. Due to the importance of the clock for many behaviours that pollinators such as bumblebees rely on to forage and pollinate efficiently, this is likely to have consequences in the field and could be a contributary factor to pollinator declines in the wild. This provides further evidence of the unpredicted consequences of neonicotinoid exposure on the beneficial insects that play a vital part in our ecosystems and food security.

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A

Appendix: Submitted Manuscript

The following manuscript was written by me and I carried out the climbing, circadian, sleep, immunohistochemistry and lethality assays for thiacloprid held within.

Neonicotinoids disrupt memory, circadian behaviour and sleep

Kiah Tasman[1], Sergio Hidalgo[1], Sean A. Rands[2], James J.L. Hodge[1,*]

Affiliations: [1]School of Physiology, Pharmacology and Neuroscience, University of Bristol Biomedical Sciences building, University Walk, Bristol, BS8 1TD, UK

[2]School of Biological Sciences, University of Bristol, Life Sciences Building, Tyndall Avenue, Bristol, BS8 1TQ, UK

Correspondence: ***james.hodge@bristol.ac.uk**

Abstract:

The effect of neonicotinoids on memory, circadian rhythmicity and sleep, all essential for efficient insect foraging and pollination, were tested in *Drosophila*. Neonicotinoids are agonists at the nicotinic acetylcholine receptors, the main mediator of synaptic transmission in the insect brain, making them potent neurotoxins and popular insecticides. Imidacloprid, clothianidin and thiamethoxam disrupted learning, behavioural rhythmicity and sleep whilst thiacloprid affected sleep. Imidacloprid and clothianidin affected neurophysiology, preventing day/night remodelling and pigment dispersing factor accumulation in the dorsal terminals of clock neurons. Knockdown of neonicotinoid-susceptible Dα1 and Dβ2 nicotinic acetylcholine receptor subunits in mushroom bodies or clock neurons caused neonicotinoid-like deficits in memory or circadian/sleep behaviour, suggesting neonicotinoid effects are mediated in these brain regions. Disruption to learning, circadian rhythmicity and sleep are likely to have detrimental effects on beneficial insects in the field.

One Sentence Summary: Neonicotinoids or knockdown of nAChR subunits reduce learning and rhythmicity, fragment sleep and prevent circadian remodelling of clock neurons.

Main text:

An estimated 84% of European crops are dependent on pollinators whose service is valued at >€22bn/year and is essential to food security(*1, 2*). However, populations of pollinating insects are declining dramatically. For instance flying insects have decreased by over 75% in Germany over the last 27 years*(3)*. Diminishing pollinator numbers are a serious threat to our food security(*2, 4*), with intensive use of insecticides being implicated in these losses(*2, 4*). However, a third of the global crop is lost to pests and without pesticides this loss could be 75%, keeping the demand for insecticides high(*5, 6*). The most common insecticides worldwide are neonicotinoids, which account for 24% of the global insecticide market valued at $1 billion/year(*6, 7*). Neonicotinoids are highly efficacious non-specific neurotoxins, affecting both target pest species such as aphids and non-target beneficial insects. They share a mechanism of action, being agonists of nicotinic acetylcholine receptors (nAChR), the main neurotransmitter in the insect nervous system. They also display target site cross-resistance in pests, diminishing their effectiveness as insecticides and unfortunately encouraging application of increasing concentrations(*7, 8*). They were branded safe compared to previous insecticides because they do not act on mammalian nAChRs(*7, 8*). However, few precursive safety tests were performed on beneficial insects, for which neonicotinoids are now known to be potent neurotoxins with well-documented lethal and sub-lethal effects(*1, 5, 7, 9, 10*). Therefore, continued intensive use is likely to have severe consequences on insect species numbers, with knock-on effects for the ecosystem, aquatic life, birds and mammals in addition to potential toxicity to humans(*1, 11-13*). Despite the 2013 European Union (EU) ban of three major neonicotinoids (the nitroimines: imidacloprid, clothianidin and thiamethoxam, the latter being a prodrug for clothiandin(*14*), neonicotinoids remain the most widely used class of insecticide globally, with a number of studies showing there has been no decrease in the quantity of banned neonicotinoids found in different populations of honey and bumble bee across Europe a year

after the ban(*15*, *16*). Furthermore, some national governments have granted multiple exemptions for the spraying of oil seed rape and a number of other applications(*17*), and neonicotinoids have high solubility and persistence in the environment(*1*). Additionally, the cyanoimine neonicotinoid, thiacloprid only came under the EU ban in 2020(*18*). Therefore, despite the current EU ban, insects are still at risk of neonicotinoid exposure.

In the field, concentrations of neonicotinoids encountered by non-target insects are typically between 1-51 μg/L for seed treated crops and 61-127 μg/L for sprayed crops(*10*). Concentrations as low as 1 μg/L (or 1 part per billion (ppb)) can cause significant behavioural effects due to the high potency of neonicotinoids, such as reduced foraging motivation in the bumblebee *Bombus terrestris*(*19*). The potential sub-lethal effects of neonicotinoids are very far-reaching because of the central role of nAChR in synaptic neurotransmission in the insect brain(*7*, *8*). Neonicotinoids cause this ligand-gated ion channel to open, thereby depolarising the neuron and increasing excitability. Prolonged exposure to the depolarising agonist may result in depolarising block, through voltage inactivation of voltage-sensitive Na^+ channels required for action potential firing and nAChR desensitization(*8*, *20*). This effect could have pronounced effects on memory formation and consolidation, which are critical for effective foraging in many pollinating insects.

Previous research in *Drosophila* demonstrated both the Kenyon cells, which constitute the insect memory centre called the mushroom body(*21*), and their output neurons(*22*), which mediate memory valence, are nicotinic with both brain regions also regulating sleep(*23*). In honeybees, sub-lethal neonicotinoids electrically inactivated(*20*) and decreased synaptic density(*24*) of mushroom body neurons and resulted in disrupted olfactory memory(*20*). Neonicotinoids also reduced honeybee antennal lobe Ca^{2+} responses and caused sensory deficits(*25*), potentially indirectly causing olfactory memory deficits.

Memory formation is also reliant on circadian rhythms(*26*) and sleep(*27, 28*).The effect of neonicotinoids on circadian rhythms and sleep is unknown. However work in *Drosophila* has shown that the setting of the central clock, synchronicity within the clock and communication between the light sensing organs and the central clock requires nAChRs signalling(*29-32*). The timing of sleep/wake cycles is also determined by the circadian clock(*33*) with the key clock neurons that mediate arousal and sleep being nicotinic(*30, 32*).

The pacemaker neurons of the insect clock consist of the pigment dispersing factor (PDF) neuropeptide expressing small and large ventral lateral neurons (s- and l-LNvs). The s-LNvs maintain rhythmicity in constant conditions and set the pace of the insect clock via PDF signalling(*34*). The LNvs are nicotinic(*29, 30*) receiving ACh from the visual circuit including the lamina, with the s-LNvs also receiving ACh-mediated light input information from the Hofbauer-Buchner (HB) eyelets. These excitatory signals regulate the electrical excitability of the LNvs required for circadian function(*35*). The LNvs are more depolarised and have an increased firing rate in the day than at night(*36*). These day/night differences in excitability helps sustain the molecular oscillation of clock genes in constant conditions as well as regulating s-LNv terminal remodelling and PDF release necessary for robust behavioural rhythmicity(*35*). The s-LNv dorsal terminals exhibit circadian remodelling, with their terminals being more branched during the day than at night(*37*) and having higher PDF accumulation in their terminals during the day than at night(*35*).

The circuitry and molecular components of the mushroom body and the clock identified in *Drosophila* and shown to be highly conserved amongst insects(*23, 38, 39*) making it a powerful model to test the effects of neonicotinoids on memory, circadian behaviour and sleep. They are also one of the insects whose nAChRs are best characterised. *Drosophila* have ten different nAChR subunits most of which are highly conserved across insect species, making it probable that a neurotoxin selected for its high potency to target insect nAChRs will affect the equivalent

nAChR in beneficial insects(*40*). Whilst the subunit conformation and location of neonicotinoid susceptible nAChRs is still largely unknown, in Drosophila the subunits Dα1 and Dβ2 have been shown to play a role in neonicotinoid susceptibility and resistance. Given the power of *Drosophila* as a model system, and the likely generalisation provided by conservation of nAChR function across insects, we tested the sub-lethal effect of field-relevant concentrations of the four major neonicotinoids on *Drosophila* memory, circadian rhythms and sleep.

Results

Field relevant concentrations of neonicotinoids cause a range of lethal and sub-lethal effects in bees(*9, 41*). In order to validate the use of *Drosophila* as a model for these lethal and sub-lethal effects, we fed field relevant concentrations of four neonicotinoids to *Drosophila* and determined their effect on longevity, offspring viability and climbing ability. As in pollinators, longevity, fecundity and mobility were all affected by neonicotinoid exposure in *Drosophila*(*1, 42-44*). The mean lifespan of control flies was 49 days while exposure to field relevant concentration of 10 µg/L clothianidin causing a reduction to 28 days, imidacloprid and thiamethoxam to 36 days, and thiacloprid, which proved the least potent, to 39 days (Extended data Fig. 1). The viability of offspring was also reduced, with 100 µg/L clothianidin, thiamethoxam or thiacloprid and 10 or 100 µg/L imidacloprid reducing the proportion of eggs that subsequently completed development and eclosed as viable adults (Extended data Fig. 2). Likewise, field relevant concentrations of the neonicotinoids imidacloprid, clothianidin and thiamethoxam (10 and 50 µg/L) all reduced locomotor performance, tested via a negative geotaxis climbing assay, whilst thiacloprid had no effect on locomotion at these concentrations (Extended data Fig. 3).

Olfactory associative memory is critical for foraging pollinators and has been shown to be disrupted by neonicotinoids in bees(*45*). In order to see if field relevant concentrations of imidacloprid, clothianidin, thiamethoxam and thiacloprid had a similar effect on flies, one-hour (h) memory (Fig. 1) was assessed using *Drosophila* olfactory shock conditioning(*46*). Imidacloprid, clothianidin and thiamethoxam all reduced memory at 10 µg/L (Fig. 1a-c) whereas thiacloprid left memory intact (Fig. 1d). Sensory controls showed that none of the neonicotinoids tested reduced the ability of flies to sense either the odours or the aversive stimuli (Extended data Fig. 4). In order to localise the effect of nAChR mis-regulation on memory, we expressed a previously validated *RNAi* specific to the neonicotinoid susceptible nAChR subunits Dα1 and Dβ2 throughout the mushroom body. Knock-down of either subunit was found to significantly reduce memory to a similar level as neonicotinoids (Fig. 1E), confirming the importance of these subunits in mushroom body mediated memory and suggesting the involvement of these subunits in the effect of neonicotinoids on this behaviour.

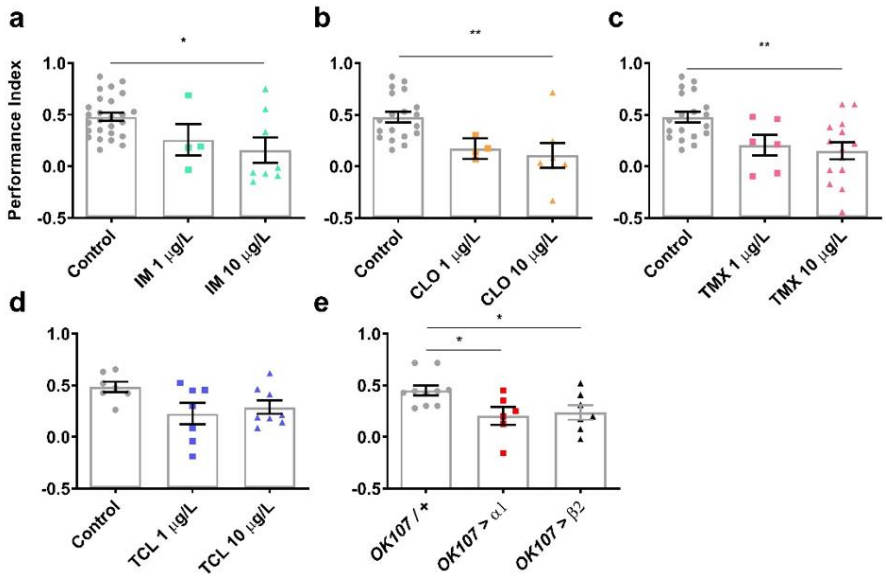

Fig. 1| Field relevant concentrations of neonicotinoids or knockdown of Dα1 or Dβ2 in the mushroom bodies reduced 1 hour memory compared to control 1h memory was reduced in flies exposed to field relevant concentrations of 1 or 10 µg/L **a**, imidacloprid (IM) (χ^2_2)=7.3, p=0.026), **b**, clothianidin (CLO) (χ^2_2=12.4, p=0.002), **c**, thiamethoxam (TMX) (χ^2_2=9.6, p=0.008) and not in **d**, thiacloprid (TCL) (χ^2_2=5.0, p=0.084). **e**, Likewise, 1h memory was reduced in flies with *RNAi* mediated knockdown of Dα1 (*OK107-Gal4>uas-nAChR-Dα1*) or Dβ2 (*OK107-Gal4>uas-nAChR-Dβ2*) throughout the mushroom body ($F_{2,20}$=4.6, p= 0.023). Each data point represents ~100 flies, n⩾4 per treatment. Graphs show mean ± standard error of the mean (SEM) (*post hoc* pairwise comparisons: $p \leqslant 0.05$*, $p \leqslant 0.01$**, $p \leqslant 0.001$***, $p \leqslant 0.0001$****). The same tests, error bars and p values were used throughout.

These results extend existing data from pollinators showing a disruption of memory formation and showing for the first time that the disruption is mediated by nAChRs in the mushroom body. Similar investigation was then carried out on sleep and circadian rhythmicity, two other behaviours that are also heavily reliant on nAChR signalling. The effect of field relevant concentrations of neonicotinoids on circadian rhythms was tested using the *Drosophila* Activity Monitor (DAM2, Trikinetics Inc, USA)(*47*). Imidacloprid, clothianidin and thiamethoxam all caused a reduction of circadian rhythmicity (Fig. 2), with flies showing greatest sensitivity to the sub-lethal circadian effects of thiamethoxam, which caused a reduction in mean rhythmicity at 1, 10 and 50 µg/L (Fig. 2d) while clothianidin and imidacloprid both caused a reduction in mean rhythmicity at 50 µg/L (Fig. 2b, c). Any concentration tested of these three neonicotinoids caused an increase in the proportion of flies that were arrhythmic (rhythmicity statistic (R.S.) ≤1.5) compared to controls (Fig. 2f-I,

Extended data Table 1). Again, thiacloprid appeared not to have sub-lethal effects, with field-relevant concentrations of the insecticide leaving circadian rhythmicity intact (Fig. 2e, i).

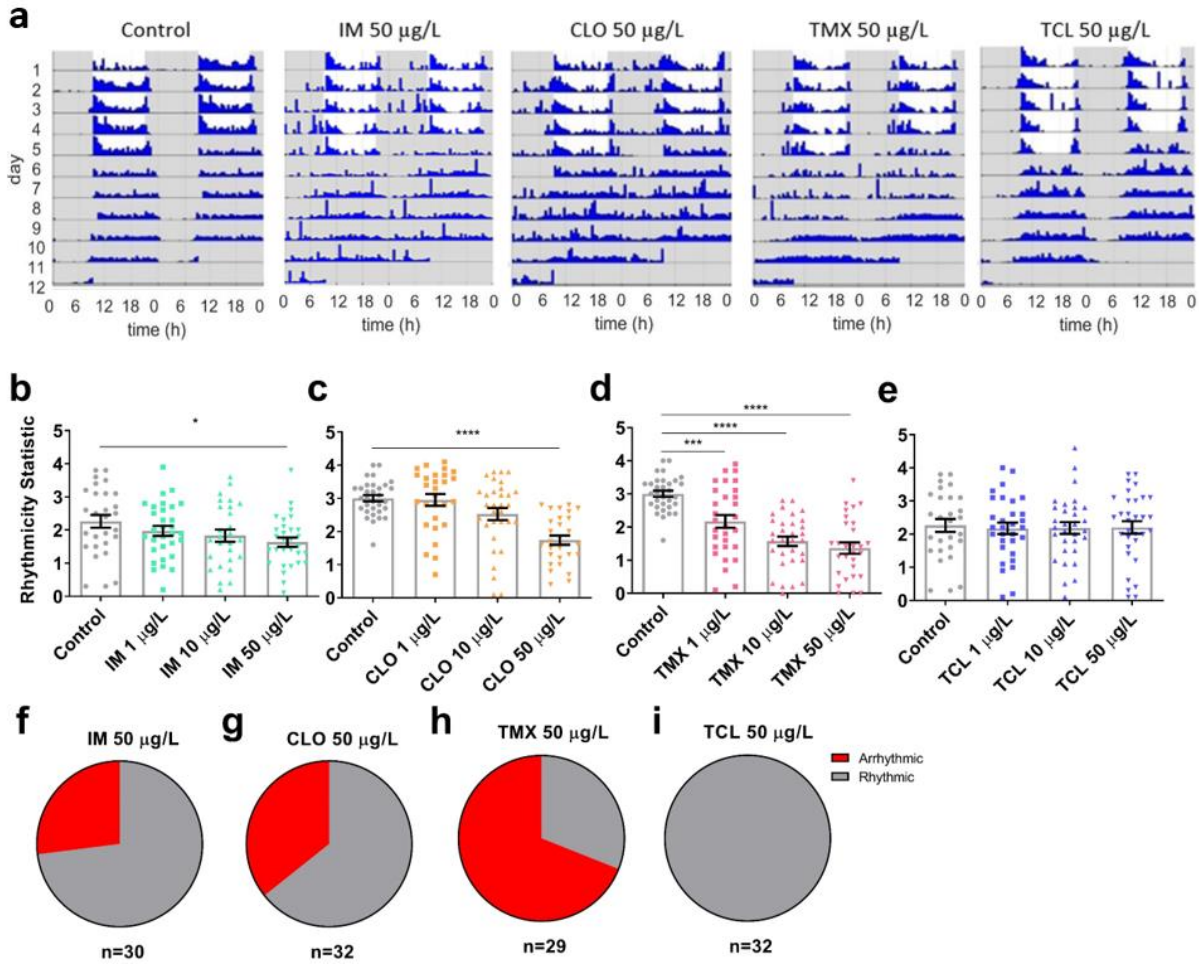

Fig. 2| Field relevant concentrations of neonicotinoids reduced behavioural rhythmicity. a, Representative actograms of the activity of single flies for control or 50 µg/L imidacloprd, clothianidin, thiamethoxam or thiacloprid. Mean rhythmicity for flies exposed to 1, 10 or 50 µg/L **b**, IM ($F_{3,112}$=2.5, p=0.06), **c,** CLO ($F_{3,116}$=14.2, p<0.001), **d,** TMX ($F_{3,118}$=23.7, p<0.001) and **e,** TCL ($F_{3,118}$=0.05, p=0.987). Each data point represents a single fly, n=28-32 flies per treatment. Pie charts showing the increase in the proportion of the population who were arrhythmic (rhythmicity statistic (RS)⩽1.5) for 50 µg/L: **f,** IM, **g,** CLO, **h,** TMX and **i,** TCL, compared to controls.

Sleep was also monitored using the DAM system, with bouts of inactivity lasting more than 5 minutes qualifying as sleep(*32*). Field relevant concentrations of all four neonicotinoids caused fragmentation of sleep, arising from sleep formed of a greater number of sleep episodes (Fig. 3b, e, h, l) of shorter length compared to control (Fig. 3c, f, I, m). This effect was greatest for clothianidin, where 1, 10 and 50 µg/L caused fragmentation of both daytime and night-time sleep (Fig. 3f, j) resulting in a reduction of night-time sleep (Fig. 3b). Thiamethoxam and

imidacloprid had a similar effect (Fig. 3e, g, i, k) but only for night-time sleep (Fig. 3a, c). Thiacloprid caused an increase in the number of episodes initiated at night (Fig. 3h) and unlike the other neonicotinoids, caused a loss in daytime sleep (Fig. 3d) at every concentration tested, due to a reduction in daytime sleep episode length (Fig. 3l). This is likely due to the increase in daytime sleep latency observed in thiacloprid treated flies (Extended data Fig. 5).

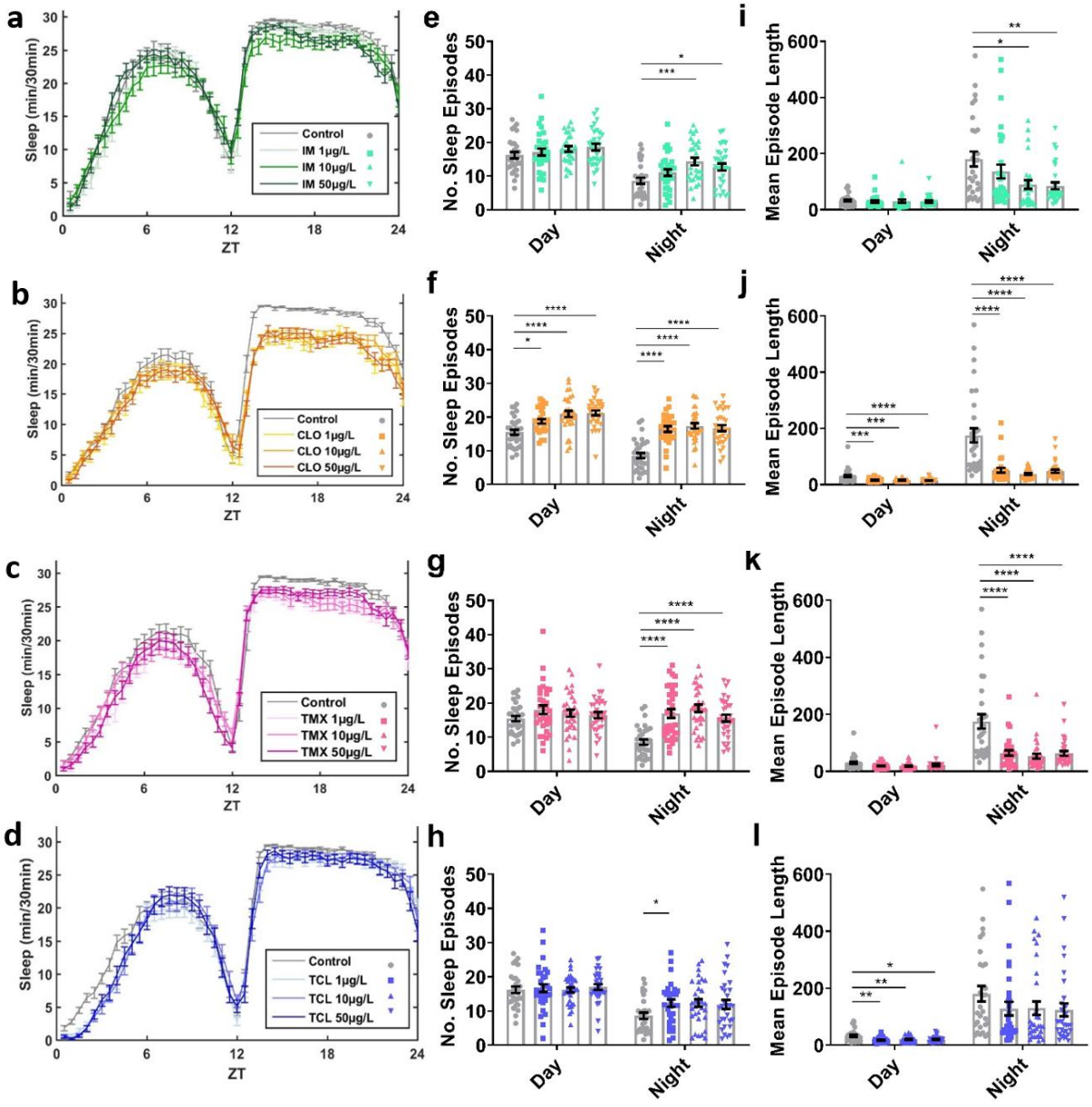

Fig. 3| Field relevant concentrations of neonicotinoids disrupted sleep behaviour. Sleep plots showing the total sleep achieved per 30 minutes bin over the 24 h period (zeitgeber time (ZT)) for flies exposed to 1, 10 or 50 µg/L of **a,** imidacloprid, **b**, clothianidin, **c,** thiamethoxam or **d,** thiacloprid. The number of (no.) of sleep episodes initiated in **e,** IM, day ($F_{3,114}$ = 1.2, p = 0.320) and night ($F_{3,114}$ = 5.5, p = 0.001), **f,** CLO, day ($F_{3,120}$ = 11.5, p < 0.001) and night ($F_{3,120}$ = 25.0, p <0.001), **g,** TMX, day ($F_{3,124}$ = 1.1, p = 0.344) and night ($F_{3,124}$ = 17.0, p <0.001) or **h,** TCL, day ($F_{3,120}$ = 0.2, p = 0.872) and night ($F_{3,120}$ = 3.0, p = 0.034). Mean length (in minutes) of sleep episodes initiated in **i,** IM, day ($F_{3,114}$ = 0.2, p = 0.889) and night ($F_{3,114}$ = 4.5, p = 0.005), **j,** CLO, day ($F_{3,120}$ = 9.9, p <0.001) and night ($F_{3,120}$ = 21.8, p < 0.001), **k,** TMX, day ($F_{3,124}$ = 2.5, p = 0.061) and night ($F_{3,124}$ = 15.7, p < 0.001) or **l,** TCL, day ($F_{3,120}$ = 5.2, p = 0.002) and night ($F_{3,120}$ = 2.0, p = 0.121). Each data point represents a single fly, n=28-32 flies per treatment.

In order to localise the effects of neonicotinoids on sleep and circadian behaviour we specifically knocked down Dα1 or Dβ2 in all clock bearing cells. This resulted in reduced behavioural rhythmicity (Fig. 4b-d, g) and shorter night-time sleep episodes (Fig. 4f, i) in Dβ2 knock downs and caused sleep to be formed of a greater number of sleep episodes in both Dα1 and Dβ2 knockdown flies (Fig. 4e, h). This again showed that loss of these subunits caused similar behavioural disruption as neonicotinoid exposure, suggesting a functional nAChR containing these subunits mediates the *in vivo* effects of these insecticides. In order to test whether this was the case, *RNAi* flies were exposed to 50 μg/L of imidacloprid or clothianidin, a concentration sufficient to reduce rhythmicity in control flies. On flies that already had their Dα1 or Dβ2 blocked genetically by expression of subunit specific *RNAi* expression throughout their clock, we found this caused no further loss of rhythmicity (Fig. 4j-k), providing evidence that the drug's *in vivo* effects were mediated through a receptor containing one or both of subunits in the clock.

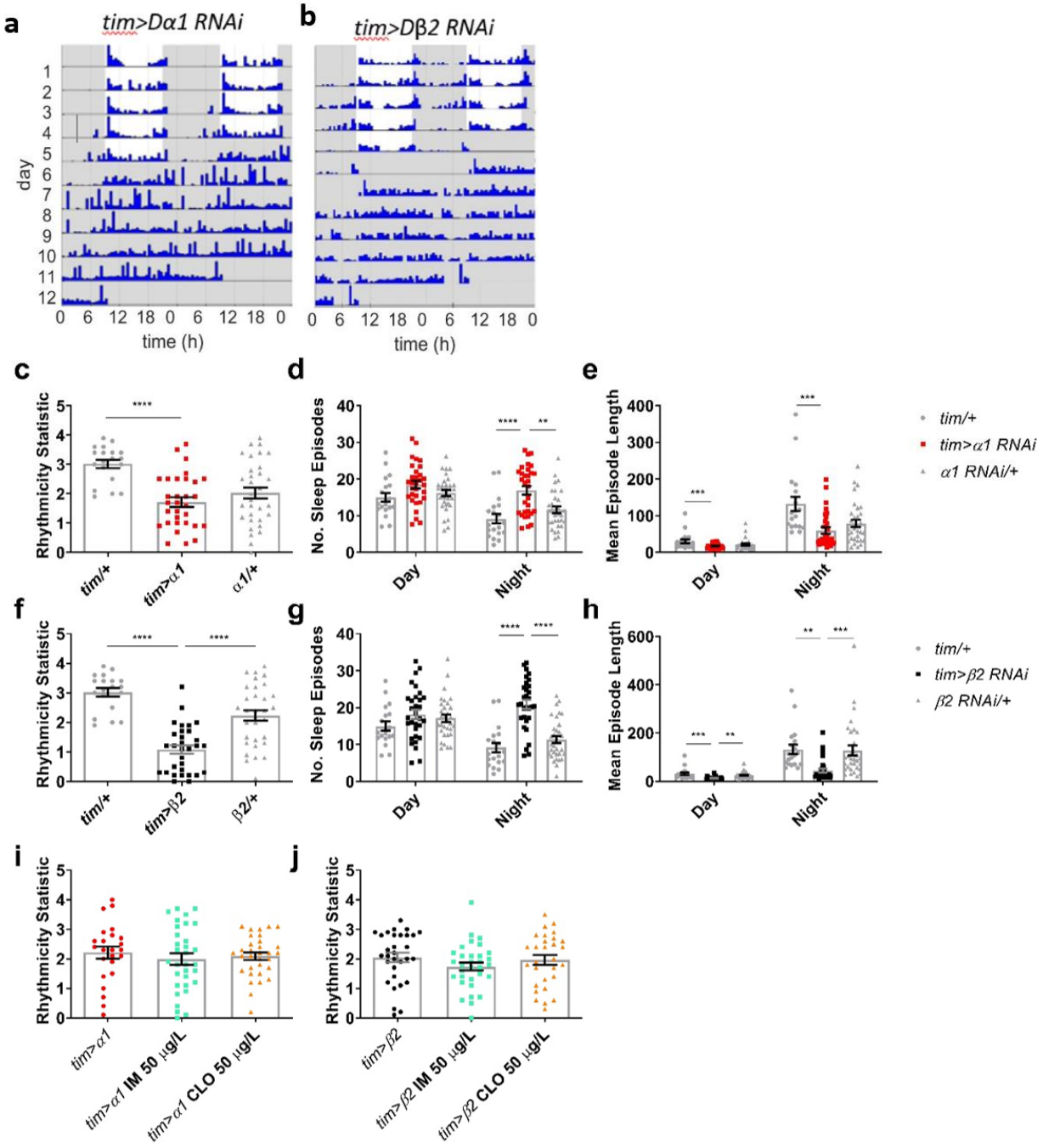

Fig. 4| Knockdown of Dα1 or Dβ2 in the clock bearing cells disrupts circadian rhythmicity and sleep with no further effect by addition of neonicotinoids. Representative actograms for **a,** Dα1 knock down (*tim-Gal4>uas-nAChR-Dα1)* and **b,** Dβ2 knockdown (*tim-Gal4>uas-nAChR-Dβ2.* Effects of knocking down *Dα1* in clock bearing cells on **c,** rhythmicity (RS) ($F_{2,79}$ = 11.8, p <0.001), **d,** number (no.) of sleep episodes in day ($F_{2,79}$ = 2.9, p = 0.063) and night ($F_{2,79}$ = 12.3, p <0.001) and **e,** mean episode length in day ($F_{2,79}$ = 5.1, p = 0.008) and night ($F_{2,79}$ = 8.3, p = 0.001). Effects of knocking down *Dβ2* in clock bearing cells on **f,** rhythmicity ($F_{2,79}$ = 31.5, p <0.001), **g,** no. of sleep episodes in day ($F_{2,79}$ = 1.6, p = 0.211) and night ($F_{2,79}$ = 28.2, p <0.001) and **h,** mean episode length in day ($F_{2,79}$ = 11.2, p<0.001) and night ($F_{2,79}$ = 9.4, p <0.001). Each data point represents a single fly, n=19-32 flies per treatment. There was no additive effect of 50 µg/L of IM and CLO on **i,** *tim>α1* ($F_{2,85}$ = 0.4, p = 0.677) and **j,** *tim>β2* ($F_{2,89}$ = 1.1, p =0.336). Each data point represents a single fly, n=24-32 flies per treatment.

To further characterise the mechanism by which neonicotinoids disrupt circadian rhythms, the circadian remodelling and PDF cycling of the sLNv dorsal terminals were investigated. As previously reported(*37*), in control flies the terminals were more branched and had higher accumulation of PDF in the day than at night (Fig. 5a-c).

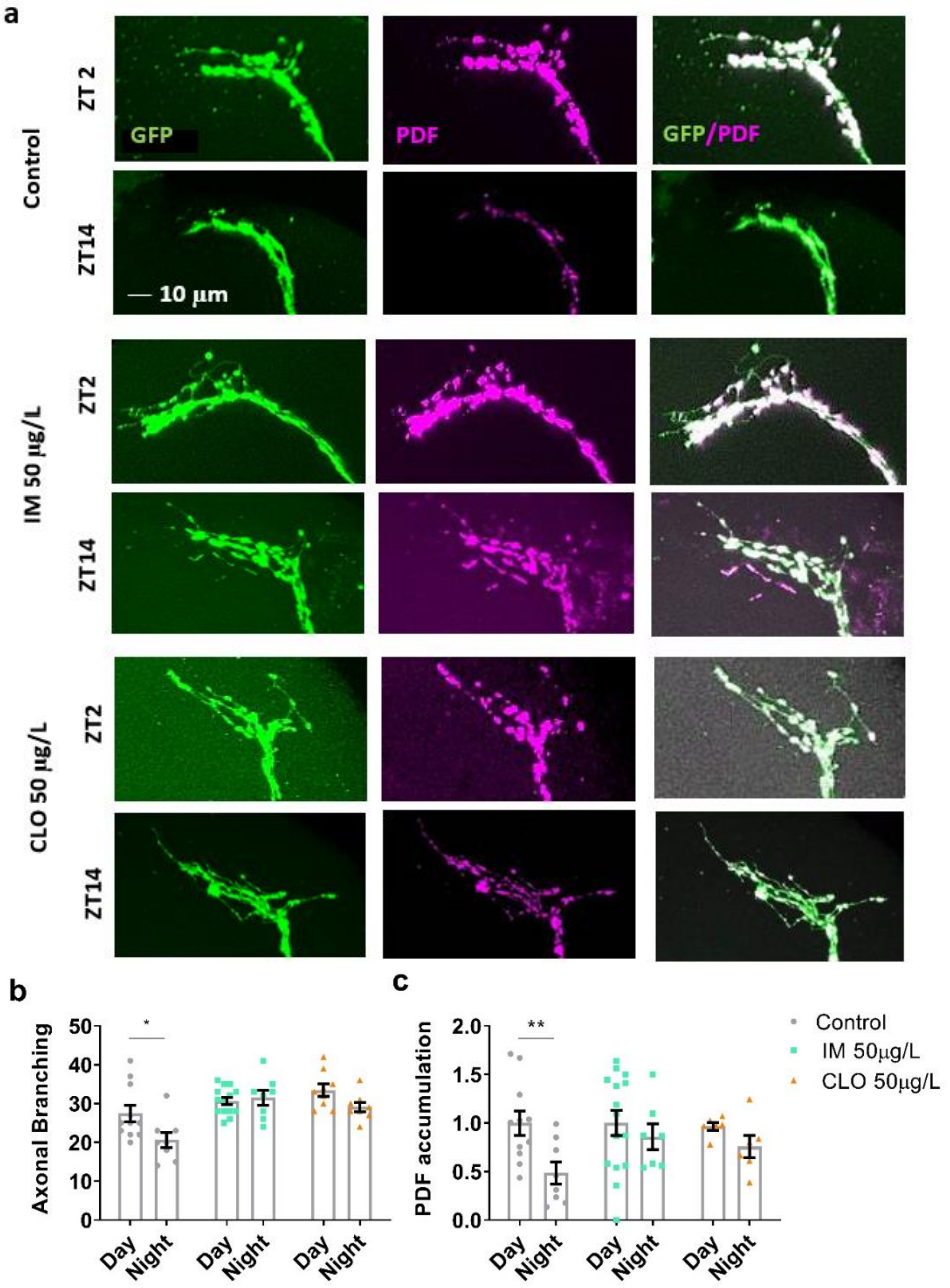

Fig. 5| Field relevant concentrations of neonicotinoids disrupt the day/night remodelling and PDF cycling in the s-LNv clock neuron dorsal terminals. a, Representative confocal images of the s-LNv dorsal terminals for control and treated (50 µg/L IM or CLO) flies in the day (ZT2 i.e. 11am) and night (ZT14 i.e. 11pm). **b,** s-LNv dorsal terminal branching complexity is greater in the day than at night for control flies (t_{17}=2.3, p=0.036). The day/night differences in complexity is removed in flies exposed to 50 µg/L of IM (t_{14}=2.1, p=0.055) or CLO (t_{15}=2.1, p=0.052). **c,** Accumulation of PDF in dorsal terminals is greater in the day than at night in control flies (t_{17}=2.9, p=0.010), treatment with 50 µg/L IM (t_{13}=1.0, p=0.332) or CLO (t_{14}=2.1, p=0.054) removed this day/night difference in PDF levels. Each data point represents a single brain, n=6-15 brains.

In contrast, flies exposed to 50 µg/L imidacloprid or clothianidin showed no difference between day and night synaptic terminal branching or PDF accumulation (Fig. 5a-c). In flies with knockdown of either Dα1 or Dβ2 nAChRs in the PDF neurons, branching and PDF accumulation again showed no difference between day and night (Fig. 6a-c).

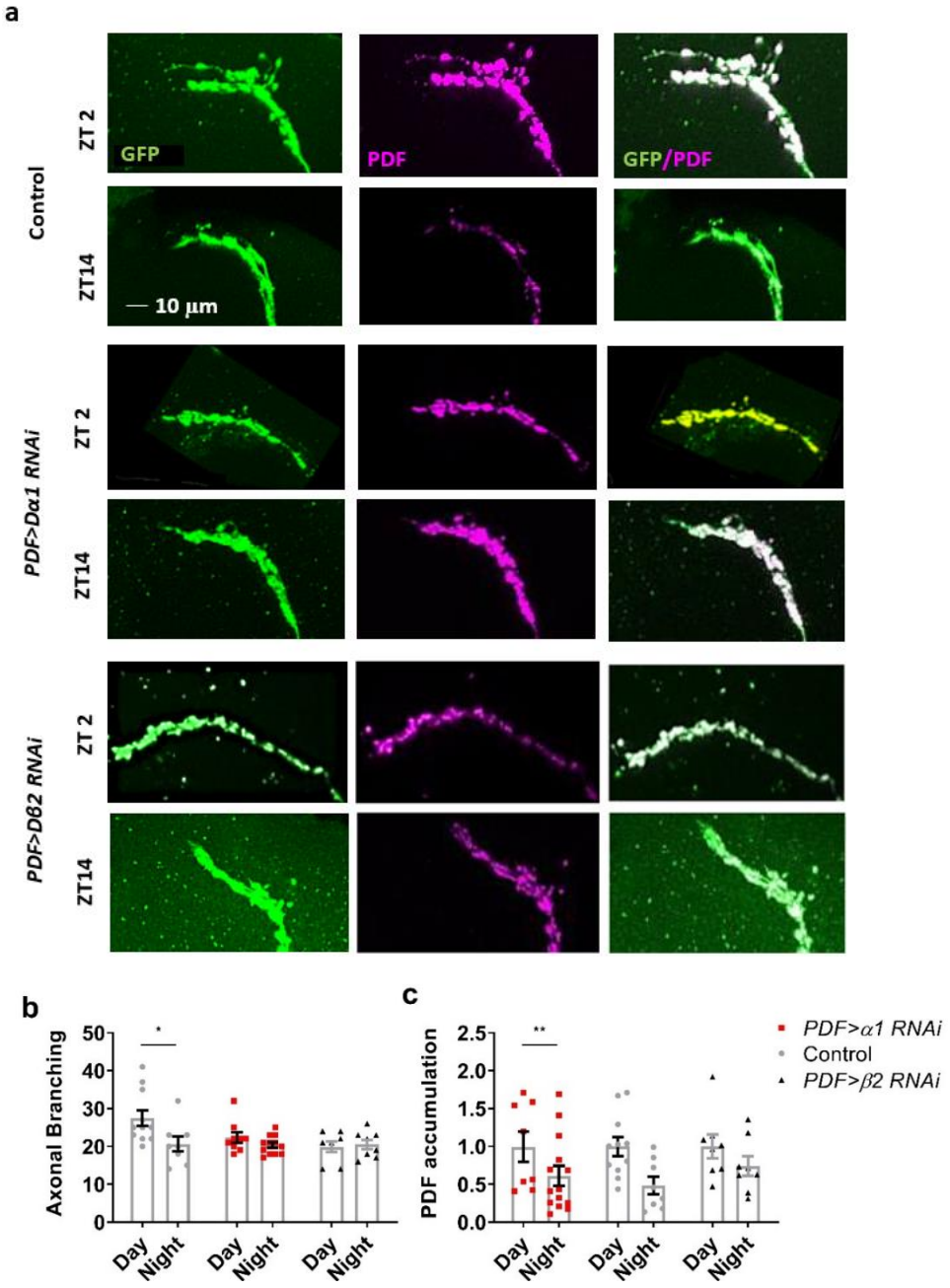

Fig. 6| Knockdown of Dα1 or Dβ2 disrupted the day/night remodelling and PDF cycling of the s-LNv dorsal terminals. a, Representative confocal images of the s-LNv dorsal terminals of control flies and flies with Dα1 (*PDF-Gal4>uas-nAChR-Dα1-RNAi*) and Dβ2 (*PDF-Gal4>uas-nAChR-Dβ2-RNAi*) knocked down in LNv clock neurons taken in the day (ZT2) and night (ZT14). **b,** The s-LNv dorsal terminals of control flies showed greater branching complexity in the day than at night (t_{17}=2.3, p=0.036), this day/night difference in terminal complexity was removed in *PDF>Dα1-RNAi* (t_{19}=1.4, p=0.183) and *PDF>Dβ2-RNAi* (t_{13}=-0.7, p=0.515) flies. **c,** PDF accumulation in the s-LNv dorsal terminals was greater in the day than at night for control flies (t_{17}=2.9, p=0.010), but not in *PDF>Dα1-RNAi* flies (t_{19}=1.8, p=0.089) and *PDF>Dβ2-RNAi* flies (t_{14}=1.3, p=0.218). Each data point represents a single brain, n=6-15 brains.

Discussion

Our data show that field relevant concentrations of all the neonicotinoids tested had some lethal effects in *Drosophila,* such as decreased viability and shortened lifespan. In contrast the behavioural or sub-lethal effects on flies differed between the neonicotinoids with imidacloprid, clothianidin and thiamethoxam disrupting memory, locomotion, sleep and circadian behaviour, while thiacloprid only caused fragmentation and reduction in sleep leaving the other behaviours intact. Thiacloprid appears to be less disruptive to the behaviours studied here than the three neonicotinoids covered by the initial 2013 EU moritorium. However its effects on sleep revealed significant sub-lethal effects even when exposure was at the lowest level reported (1 ppb), in addition to causing decreased viability and early death, providing strong evidence in support of the EU's recent decision to extend the neonicotinoid ban to thiacloprid. Clothianidin and thiamethoxam showed the greatest effects, which is consistent with them being full agonists at nAChRs and many other studies in pollinators finding them to be more toxic and potent than the partial agonist imidacloprid(*7, 41, 48*).

The effects of field-relevant concentrations of neonicotinoids on memory in *Drosophila,* reiterates the conserved toxicity and sub-lethal effects of the insecticides on non-target insect species. That neonicotinoid exposure did not affect the ability of flies to sense the stimuli or respond to reinforcement, confirms that the neonicotinoids interfere with memory formation itself. This was supported by the data showing that knockdown of the neonicotinoid susceptible Dα1 or Dβ2 subunits in just the mushroom body was sufficient to cause the memory deficits observed in the neonicotinoid exposed flies. The Kenyon cells of the mushroom body are cholinergic(*48*), with many of the projection neurons bringing olfactory information from the glomeruli of the antennal lobe forming nicotinic synapses onto the mushroom body(*49*) and mushroom body to mushroom body output neuron synapses signalling via nAChRs(*22*). Therefore, neonicotinoids can act at multiple nAChR synapses in the mushroom body circuit,

disrupting the plasticity-relevant signals for memory formation. Furthermore in bees, field relevant concentrations of neonicotinoids disrupted mushroom body-mediated olfactory memory, electrically inactivated Kenyon cells(*20*), decreased their synaptic density(*24*) and reduced antennal lobe Ca^{2+} responses upstream of the mushroom body(*25*).

Similarly, the sleep and circadian effects caused by neonicotinoid exposure appear to be due to the neonicotinoids acting directly upon the clock. Knockdown of Dβ2 nAChR subunit in the clock bearing cells resulted in the same disruption of rhythmicity as exposure to field-relevant concentrations of neonicotinoids, whilst knockdown of Dα1 or Dβ2 caused changes to sleep behaviour reflecting those seen in neonicotinoid exposed flies. This suggests that Dα1 and Dβ2 mediate the effects of neonicotinoids on clock bearing cells, bringing about the disruptions in circadian rhythms and sleep. Consistent with this, exposure of Dβ2 knockdown flies to imidacloprid or clothianidin caused no further effect on circadian rhythmicity confirming that Dβ2 in clock neurons mediates the *in vivo* effect of the neonicotinoids on circadian rhythms.

Given that the LNvs are nicotinic, neonicotinoids may be acting via these pacemaker neurons, which usually receive excitatory ACh inputs from light-sensing organs(*29, 30*). The electrical state of these neurons influences their circadian output including the circadian remodelling of the s-LNv dorsal terminals and circadian abundance of PDF(*35, 50*), with the release of this neuropeptide being necessary for behavioural rhythmicity(*34*). We found that neonicotinoid exposure caused a loss of PDF cycling and terminal plasticity, with the terminals remaining in a branched, day state continuously, suggesting that the depolarising block effect of neonicotinoids can remove the normal day-night changes in the electrical state of the neurons required for circadian rhythms. Indeed, nAChR synaptic signalling is required for the rhythmic firing of action potentials clock neurons in *Drosophila* and other insects(*31, 51, 52*). Knock-down of the neonicotinoid susceptible nAChR subunits Dα1 or Dβ2 also removed day-night differences in the terminals as has been observed for flies with electrically silenced LNvs(*50*).

The agonist action of neonicotinoids on nAChRs on the LNvs may also explain the disruption to sleep behaviour. The electrical state of the l-LNvs is vital to their role as arousal neurons, with hyperexcitation of the l-LNvs leading to sleep defects such as loss of night-time sleep and shorter sleep episodes(*53*) which occurred in the neonicotinoid exposed flies.

The high degree of structural and functional conservation of nAChRs between flies and bees(*7, 40, 54*), and the conserved lethal and sub-lethal effects of reduced viability, longevity, locomotion and memory we demonstrated in flies as reported in bees suggest that the novel sleep and circadian disruptions we observed are also likely to occur in beneficial insects in the field(*1, 42-45*). Previous work has shown that sub-lethal effects observed in the lab translate to the field, for example neonicotinoid reduced foraging motivation observed for bumblebees both in the lab and in free flying colonies in the field(*19, 55*). Reduced behavioural rhythmicity is likely to reduce the amount of activity carried out in the daytime, reducing pollination and foraging opportunities(*56*). The reduction in total sleep and fragmentation of sleep will reduce the quantity of deep sleep achieved, as deep sleep occurs later into the sleep episode(*57*). As deep sleep is particularly important for memory consolidation(*27*), this may compound the direct effects of neonicotinoids on memory, which will again impact foraging efficiency.

In summary, nAChR subunits Dα1 and Dβ2 expression in the mushroom body appears to mediate the effect of field-relevant concentrations of imidacloprid, clothianidin and thiamethoxam on memory, while expression of these subunits in clock bearing cells mediates the effect of neonicotinoids on circadian rhythmicity and sleep. Thiacloprid was less toxic than the other three insecticides, only disrupting sleep, which seems to be the most sensitive behavioural metric of the sub-lethal effects of neonicotinoids. In addition, all four of the neonicotinoids tested decreased both viability and shortened lifespan, therefore supporting their continued ban in the EU and the extension of this ban to cover thiacloprid. This work illustrates the utility of neonicotinoids as a pharmacological tool for exploration of nAChR

function, as well as the use of *Drosophila* in revealing the mechanism of action of neonicotinoids and elucidating the sub-lethal and lethal effects of these insecticides, highlighting their potential impact on insects in the field.

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Extended Data

Materials and Methods

Fly husbandry and genotypes

The following fly stocks were used: wild-type strains *iso31* (Gift from Dr Ralph Stanewsky, University of Münster, Germany) for climbing, circadian and sleep assays and *CSw-* (gift from Dr Scott Waddell, University of Oxford, UK) for all other experiments, *Pdf-Gal4* (Bloomington *Drosophila* stock center number (BDSC): 6900), *elav-Gal4* (BDSC: 8760), *tim-Gal4*[27] (gift were from Dr Ralf Stanewsky, University of Münster, Germany), *OK107-Gal4* (BDSC: 854), *uas-mcd8::GFP* (BDSC: 5137), *uas-nAChR-Dα1-RNAi* (BDSC: 28688), and *UAS-nAChR-Dβ2-RNAi* (BDSC: 28038) validated in(*22*). For all experiments, flies were collected shortly after eclosion and used within 5 days. For climbing, circadian, sleep, longevity, immunohistochemistry and offspring survival assays females were used, for learning and memory mixed sex groups were used.

Flies were bred, maintained and tested on standard polenta-based food mixture at 25^{o}C, 55-65% humidity under 12h light:12h dark (LD) conditions. Food was made up in 5 L quantities and contained: 400 g polenta, 35 g granulated agar, 90 g active dried yeast, 50 g soya flour, 400 ml malt extract and 200 ml molasses, with 40 ml of propionic acid (Sigma-Aldrich, #94425) and 100 ml of nipagin (Sigma-Aldrich, #H5501) added once cool. Neonicotinoids were added to food before it set from a frozen and aliquoted stock solution of 100,000 μg/L ddH_2O. The neonicotinoids were analytical standard (PESTANAL Sigma-Aldrich): imidacloprid, clothianidin, thiamethoxam, and thiacloprid.

Longevity

Ten once mated, one day old females were placed in a vial containing control or neonicotinoid containing food and transferred into a fresh vial every 2 days with the number of dead flies noted. This was continued until all flies were dead(*58*) with ten repeats being performed per treatment group. A survival curve was created and analysed using GraphPad (GraphPad Prism version 6.05 for Windows, GraphPad Software) and mean lifespan calculated. The difference of the treatment survival curve from the control survival curve was analysed using a log-rank (Mantel-Cox) test.

Offspring viability

Flies were reared on control or neonicotinoid containing food. Ten groups of ten once mated females were collected, and then each female was placed in a vial of control fly food and allowed to lay eggs over a 24hour period. The number of eggs in each vial was quantified and then compared to the number of adult flies which successfully eclosed from the vial ~15 days later, giving a % offspring survival for each group(*59*).

Locomotor assay

Climbing ability was used as a measure of locomotion of adult flies and was determined by the negative geotaxis assay, whereby flies were tapped to the bottom of a tube, causing them to move away from gravity (negative geotaxis). Twenty-five groups of ten females were placed in vials of control or neonicotinoid containing food for 5 days. They were then placed into empty vials. After 5 minutes of acclimatisation, flies were knocked to the bottom of the vial and given 10 seconds to climb(*60*). The performance index represents the proportion of flies who successfully climbed ≥7 cm in 10 seconds.

Aversive olfactory conditioning

1h memory was tested using aversive olfactory conditioning(*46*). Groups of 30-50 one-five days old mixed sex flies were reared on control or neonicotinoid containing food. Flies were exposed consecutively to one of two odours, either 4-methylcyclohexanol (Sigma) or 3-octanol (Sigma) diluted 1:500 and 1:250 respectively, paired with 1.5 second pulses of 70 V electric shock, with 3.5 second pauses between shocks. Flies were then returned to food vials for 1 hour before memory was tested. For testing, flies were loaded into the choice point of the T-maze and, after a 90 second acclimatisation period, were given the choice of two tubes, one containing each of the test odours. Flies were given 2 minutes and then the proportion in each arm was counted. A separate group of flies were then trained and tested with the reciprocal odour. The performance index score represents the proportion of flies who correctly avoided the arm containing the odour which had been delivered with shock during training, as shown below.

PI = (number of correct flies−number of incorrect flies)/total number of flies.

The PI for flies shocked with each odour separately were averaged to give an n=1.

Sensory Controls

Sensory controls were carried out to check the capacity of treatment groups to sense olfactory and shock cues(*46*). For olfactory acuity, groups of 1-5 day old 30-50 mixed sex flies, reared on control or neonicotinoid food, were loaded into the T-maze and provided with a choice between an odour (1:500 4-methylcyclohexanol or 1:250 3-octanol) and fresh air. For shock reactivity, similar groups of flies were given a choice between two shock tubes, one of which was delivering 1.5 second pulses of 70 V electric shock, with 3.5 second pauses between shocks. In both cases, flies with normal sensory capacity should avoid the stimuli. The proportion of each group who avoided the odour or shock was reported.

Circadian rhythms

Behavioural rhythmicity data was collected using *Drosophila* Activity Monitors (DAM2, TriKinetics Inc)(*61*). Virgin females were placed in individual tubes in the DAM, with control or neonicotinoid containing food, 32 flies per treatment, for 5 days in LD followed by 5 days constant darkness (DD). Flies who died before the end of day ten were not included in analysis. DAM tubes were intersected with an infrared beam, with each beam cross counted as an activity bout.

To quantify circadian rhythmicity, the data was summed into 30 minutes bins. From this an actogram was created and rhythmicity statistic and period length were calculated for each individual fly for the DD portion using Flytoolbox(*62*) in MATLAB (MATLAB and Statistics Toolbox Release 2012b, The MathWorks). The proportion of flies in each treatment group whose Rhythmicity Statistic was below 1.5, generally considered to denote arrhythmicity(*62*), was calculated. This was then displayed in a pie chart for each group, after being normalised by removing the proportion of controls who were arrhythmic for each run.

Sleep

For analysis of sleep behaviour, flies were loaded into the DAM as described above. Sleep measures were extracted from activity data from 5 days of LD, summed into one minute and 30 minutes bins. Sleep was defined as bouts of inactivity lasting more than 5 minutes, as is convention(*62, 63*). The mean total sleep, mean sleep episode length, mean number of sleep episodes per day and night and mean sleep latency were calculated for each individual using the Sleep and Circadian Analysis MATLAB Program (SCAMP) in MATLAB(*64*). For each treatment, a mean sleep profile was also plotted showing mean sleep quantity per 30 minutes bin over the 24 h period.

Measuring the arborisation and PDF cycling of s-LNv dorsal terminals

Immunohistochemistry was adapted from the method described in Fernández et al(*37*). Virgin females were collected and placed in vials. After 5 days LD, on control or neonicotinoid food, flies were anesthetised using CO_2 at either 2h after lights on (ZT2) or 2h after lights off (ZT14) and decapitated. Heads were fixed in phosphate-buffered solution (PBS) with 4% paraformaldehyde (Thermo Fisher Scientific) containing 0.008% Triton X-100 (Sigma-Aldrich) for 45 minutes at room temperature. Heads were washed quickly twice in PBS with 0.5% Triton X-100 (PBT 0.5%), followed by three 20 minutes washes in PBT 0.5% and dissection in PBT 0.1%. Brains were blocked in 5% Normal Goat Serum (NGS, Thermo Fisher) for 1h, then incubated for 36 hours at 4ºC with mouse monoclonal anti-PDF (Developmental Studies Hybridoma Bank, #PDF-C7) and rabbit polyclonal anti-GFP (Life Technologies # A11122) in NGS at concentrations of 1:200 and 1:1000 respectively.

Brains were washed again as before and then incubated for 3h at room temperature followed by 24h at 4 °C with Alexa Fluor Plus 488 Goat anti-mouse (Life Technologies # A32723) and Alexa Fluor Plus 555 goat anti-rabbit (Life Technologies # A32732) in NGS at concentrations of 1:1000 and 1:100 respectively. Brains were washed once more and then mounted onto glass slides using spacers (SecureSeal™, Grace Bio-Labs #654002), covered with VectaShield hard set medium (Vector Laboratories) and secured with CoverGrip (Biotium #23005).

Imaging was carried out on a Leica SPE confocal laser scanning microscope with the green channel imaged at 480–551 nm and the red at 571–650 nm. Z stacks were captured of the s-LNv dorsal terminals using a 64x oil immersion objective, with a step size of 2 µm. Maximal projection stacks were created and analysed using FIJI (ImageJ) (*65*). The arborisation of each s-LNv terminal was calculated using an adaptation of the Scholl analysis(*37*). Six concentric rings 10 µm apart were drawn, centred at the first branching point and the number of branches touching each ring was summed. Both hemispheres were measured. Mean scores for ZT2 and ZT14 in each group were compared using Pearson's *t*-test using SPSS Statistics 24 (IBM Corporation).

For PDF staining intensity, the image was cut at the first branching point to create an image containing only the terminals and not the cell axon and the image was analysed in FIJI (ImageJ). The image was transformed into 8-bit and the threshold adjusted to create a black and white image. The despeckle filter was used to reduce noise and watershed segmentation carried out to separate the different PDF compartments. This image was then used as a template for calculating the PDF staining in the original maximal projection image, allowing the PDF staining intensity to be calculated for each of these compartments and the mean taken. The mean for both hemispheres of the brain was calculated and reported, and the means for ZT2 and ZT14 were compared as for axonal branching.

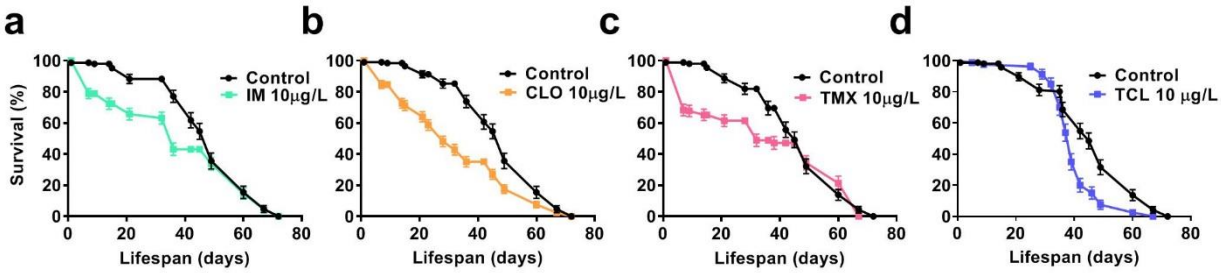
Statistical analysis

Normality of the data was checked using a Shapiro-Wilk test. Data were also checked for homogeneity of variance using Levene's test for equality of variances. Unless otherwise stated, means were then compared using a one-way ANOVA with *post hoc* pairwise comparisons being carried out using Tukey's multiple comparisons test. Where data were not normally distributed, a Kruskal-Wallis one-way ANOVA was carried out with Dunnett's multiple comparison. Statistical analysis was done in SPSS Statistics 24 (IBM Corporation). Graphs were created in GraphPad (Prism version 8.0.0 for Windows, GraphPad Software).

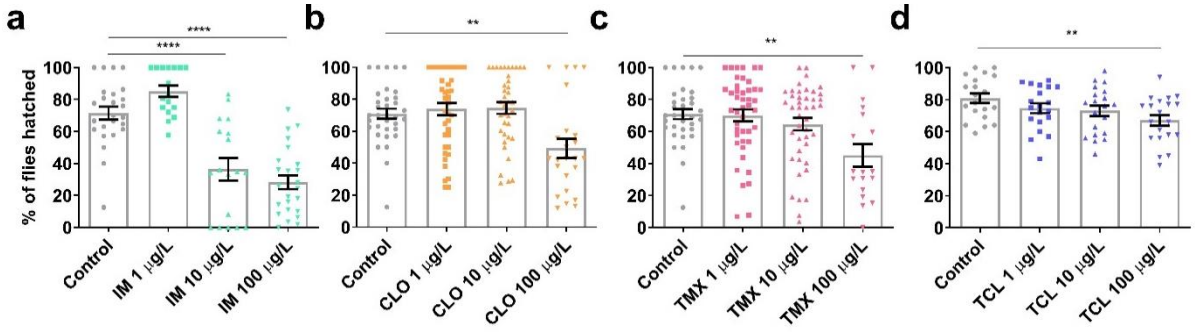
The sleep data failed the assumptions of normal distribution and homogeneity of differences. Thus, permutation tests were conducted in R 3.4.1. As the resulting *p* values closely matched

those produced by analysing the same data using a one-way ANOVA as above, and because ANOVA is relatively robust to deviations from normality when sample sizes are large, the results of the one-way ANOVA were displayed.

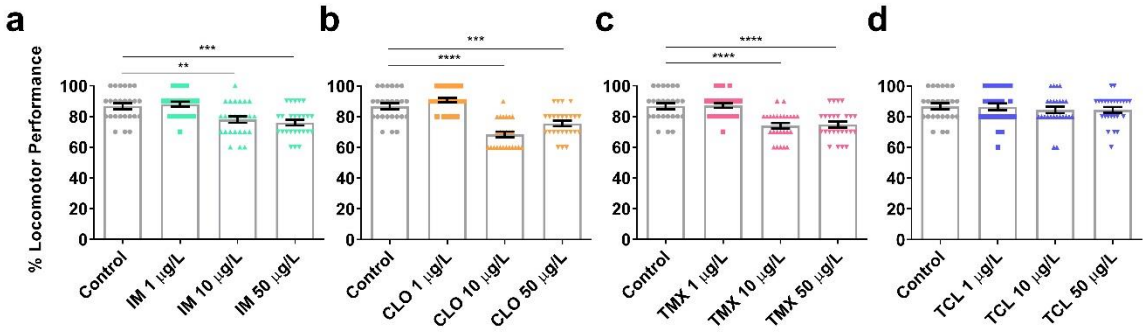
Extended Figures 1-5



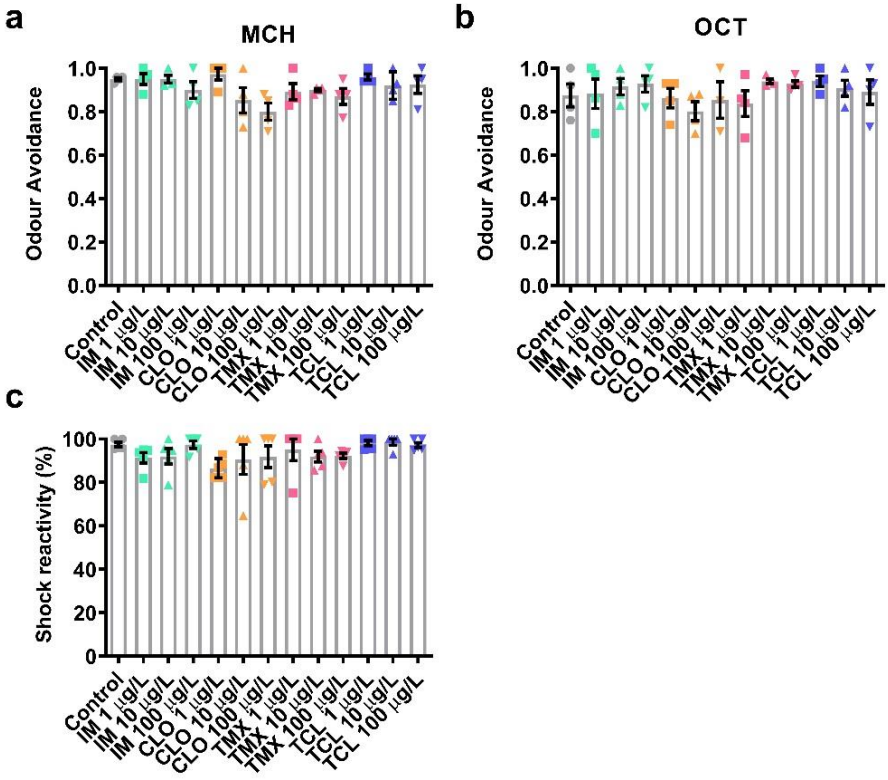
Extended data Fig. E1| Field relevant concentrations of neonicotinoids reduced longevity. Compared to the median lifespan for control flies (49 days) flies exposed to 10 µg/L **a,** IM (36 days,(χ^2_1=8.3, p=0.004), **b,** CLO (28 days, (χ^2_1=41.0, p <0.001), **c,** TMX (36 days, (χ^2_1=5.8, p=0.016) and **d,** TCL (39 days, (χ^2_1=18.5, p <0.001) had shorter lives. n=100 flies for each group.



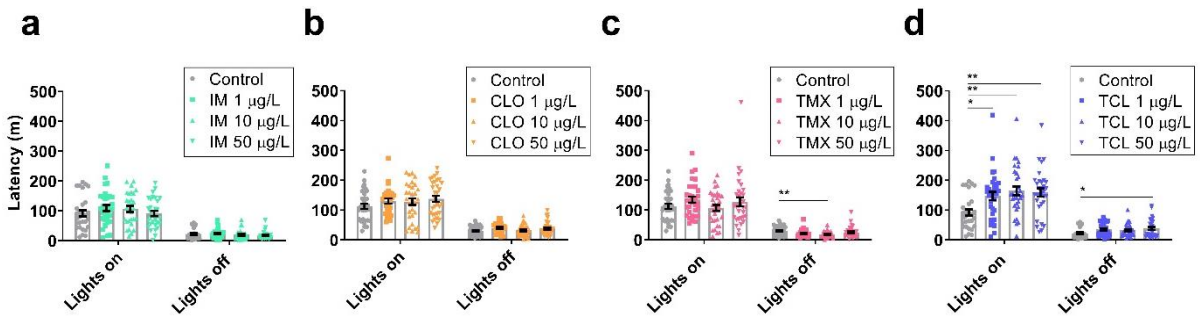
Extended data Fig. E2| Field-relevant concentrations of neonicotinoids reduce viability. The viability of offspring of flies exposed to 1, 10 or 100 µg/L **a**, IM ($F_{3,80}$ = 31.7, $p \leq 0.001$), **b,** CLO ($F_{3,132}$ = 6.9, p <0.001), **c,** TMX ($F_{3,135}$ = 5.4, p = 0.002) and **d**, TCL ($F_{3,76}$ = 3.8, p = 0.014), n=10 groups of 10 once mated female flies for each treatment. Viability was measured by counting the number of eggs laid by 10 once mated female in 24 h period and then counting the % of those eggs that completed development, eclosing viable adults ~15-18 days later.



Extended data Fig. E3| Field relevant concentrations of neonicotinoids reduce locomotor performance. Locomotor performance was measured using the negative geotaxis climbing assay, flies exposed to 10 or 50 µg/L of the banned neonicotinoids: **a,** IM ($F_{3,96}$ = 9.9, p <0.001), **b,** CLO ($F_{3,96}$ = 32.0, p <0.001), **c,** TMX ($F_{3,96}$ = 15.8, p <0.001) significantly reduced climbing performance, while the non-banned neonicotinoid **D)** TCL ($F_{3,96}$ = 0.4, p = 0.762) did not affect locomotion. n=25 flies for each treatment group.



Extended data Fig. E4| Field relevant concentrations of neonicotinoids do not disrupt olfaction or shock reactivity. Sensory controls for olfactory-shock conditioning memory assays (Fig. 1) show 1, 10 and 100 µg/L IM, CLO, TMX and TCL did not affect **a,** odour avoidance of 4-methylcyclohexanol (MCH) (χ^2_{12} = 19.5, p = 0.076), or **b,** 3-octanol (OCT) (χ^2_{12} = 10.0, p = 0.674) and **c,** shock reactivity (χ^2_{12} = 22.6, p = 0.031). Each data point represents a group of ~50 flies, tested together, n=4 for each group.



Extended data Fig. E5| Field relevant concentrations of thiacloprid increase daytime sleep latency. The mean latency in minutes (m) before sleep was initiated after lights on or lights off, for flies exposed to 1, 10 or 50 µg/L **a,** IM, day ($F_{3,114}$=0.9, p=0.441) and night ($F_{3,114}$=0.9, p=0.468), **b,** CLO, day ($F_{3,120}$=1.1, p=0.333) and night ($F_{3,120}$=2.0, p=0.124), **c,** TMX, day ($F_{3,124}$=1.3, p=0.264) and night ($F_{3,124}$=4.3, p=0.007) or **d,** TCL, day ($F_{3,120}$=5.6, p=0.001) and night ($F_{3,120}$=2.7, p=0.50). Each datapoint represents a single fly, n=28-32

Extended Table E1

Extended data Table E1| Field relevant concentrations of neonicotinoids increase the proportion of the population (%) exhibiting arrhythmicity compared to controls

	IM	CLO	TMX	TCL
1 µg/L	8%	11%	10%	1%
10 µg/L	19%	19%	23%	4%
50 µg/L	27%	36%	65%	0%

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